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# Biological and Physical Characterization of Aerosols Generated in Showers

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# Biological and Physical Characterization of Aerosols Generated in Showers

**by**

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**Thesis**

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

**Master of Science in Engineering**

**The University of Texas at Austin**

**May, 2014**

# Acknowledgements

Funding for this research was provided by the National Science Foundation Graduate Research Fellowship Program under Grant Number DGE-1110007, and the American Water Works Association Holly A. Cornell Scholarship. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Science Foundation.

I would like to thank my advisors, Dr. Kerry Kinney and Dr. Mary Jo Kirisits for their support and guidance during this project. I would also like to thank everyone in the Environmental and Water Resources Engineering Program for their support and friendship during my time at the University of Texas at Austin.

# Abstract

## Biological and Physical Characterization of Aerosols Generated in Showers

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The University of Texas at Austin, 2014

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Although drinking water treatment facilities treat raw water sources to remove and inactivate microorganisms, some microorganisms will survive treatment and enter the distribution system. Regrowth and infiltration in distribution systems leads to the presence of diverse microbial communities in drinking water at residential taps. In the shower environment, these microorganisms might be present in the bulk water, within biofilms in the plumbing or on shower surfaces. Shower microbial communities are important to investigate because this environment might subject the occupant to microbial exposure risks through inhalation, ingestion, and skin contact. The goal of this research was to investigate the biological and physical characteristics of aerosols generated during shower operation. Specifically, this research sought to (1) identify potentially pathogenic and allergenic microbial species in residential showers, (2) determine the contribution of tap water to shower bioaerosols, and (3) determine the effects of air exchange rate and shower water temperature on shower bioaerosols, particulate concentrations, relative humidity, and ambient temperature.

This study yielded several key findings. The allergenic fungal species *Alternaria alternata* was detected in shower aerosols in a residential shower in Austin, TX, and in a residential shower in San Antonio, TX, but it was not found in water or shower surface biofilms from these two showers. In addition, *Mycobacteria* was found in the Austin, TX, residential shower. However, quantitative real-time polymerase chain reaction (qPCR) indicated that the opportunistic human pathogens that make up the *Mycobaterium avium* complex were not present at this residence to the detection limit of 29 gene copies/µL.

Experiments run in a recirculating experimental shower indicate that tap water is more diverse and contains substantially different communities than do shower bioaerosols. However, it was not evident from the experiments conducted whether tap water significantly impacted the composition of the bioaerosol communities present during shower operation. Nevertheless, particle concentration monitoring suggests that shower occupants are exposed to a 10× increase in inhalable particulate concentrations during shower operation as compared to before shower operation. Additionally, the concentration of particles in air during shower operation increases with decreasing air exchange rate. Finally, it was determined that increasing shower water temperature from 25 to 40˚C increased the relative humidity in the shower unit by over 10% and ambient temperature by ~5˚C during shower operation. The work reported in this thesis adds to our understanding of the shower microbiome and provides suggestions for further research on the topics presented.

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# CHAPTER 1. INTRODUCTION

## 1.1 Introduction

Municipal drinking water treatment facilities treat raw water sources to remove contaminants and inactivate potentially harmful microorganisms. However, it is infeasible and cost prohibitive for these treatment facilities to remove all of the microorganisms from source waters, and, as a result, some microorganisms might be present in the treated water. In addition, infiltration and regrowth in drinking water distribution lines leads to the presence of diverse microbial communities before this water reaches the consumer (Ingerson-Mahar et al., 2012). Indeed, many studies have shown diverse microbial communities in municipal tap waters (Berry et al., 2006; Chowdhury, 2012; Gonçalves et al., 2006; Henne et al., 2012; Holinger et al,. 2013; Lu et al., 20130; Vesper et al., 2007; White et al., 2011). Few studies, however, have investigated the microbial communities present in residential showers (Falkinham et al., 2008; Feazel et al., 2009; Hamada et al., 2010; Kelley et al., 2004). It is important to consider microbial communities in residential showers because this environment might subject the occupant to microbial exposure risks through inhalation, ingestion, and skin contact. This study focuses on characterizing the microorganisms present in aerosols generated during shower operation and investigates several factors that might affect the composition and concentration of the microorganisms released.

## 1.2 Objectives

This research aims to add to the existing literature regarding the shower microbiome by investigating shower aerosols in two different settings: residential showers and a controlled experimental shower set-up. The specific objectives of this research are (1) to detect the presence and potential sources of allergenic and opportunistic human pathogens in residential showers (Section 4.1), (2) to delineate the microbial assemblages aerosolized from tap water in showers (Section 4.2), and (3) to determine the effects of air

exchange rate and shower water temperature on shower bioaerosols, particulate concentrations, relative humidity, and ambient temperature (Sections 4.2 and 4.3).

To address these objectives, air, water, and biofilm samples were collected from residential showers in Austin, TX, and in San Antonio, TX. These samples were then used to look for the presence of specific allergenic fungal species and opportunistic human pathogenic species in the two residential showers. To the author's knowledge, this is the first study to investigate specific fungal aerosols in a shower environment. Additionally, a controlled experimental shower set-up was used to collect air and water samples before and during shower operation over a variety of air exchange rates and water temperatures. This chamber also was used to collect particle size distribution data, relative humidity data, and ambient temperature data over a range of air exchange rates and water temperatures.

# CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

## 2.1 Microorganisms in Residential Tap Water

Municipal drinking water in the United States is treated using techniques such as coagulation and flocculation, filtration, and disinfection to remove and inactivate microorganisms. However, not all microorganisms are removed from source water before it is distributed to the consumer. Instead, microorganisms are removed to the extent considered safe to drink (as determined by monitoring indicator organisms). As a result, microorganisms are present in the effluent of drinking water treatment facilities. Drinking water is tested for indicator organisms (e.g., coliforms such as *Escherichia coli*) as a surrogate means of assessing pathogen removal. However, the use of indicator organisms has important drawbacks. Indicator assays do not test for specific pathogens; rather they test for organisms whose presence are indicative of pathogen contamination, and, as such, a positive result might lead to an unnecessary treatment response (Ingerson-Mahar et al., 2012). Additionally, indicator organisms are associated with fecal contamination and might not indicate the presence of non-fecal microorganisms capable of causing illness (Ingerson-Mahar et al., 2012). Due to the use of indicator bacteria assays, it is possible for pathogens to survive treatment and make it into distribution systems undetected. The current standard for the prevention of pathogen contamination in drinking water as set by the 1989 Total Coliform Rule is that a public water system must not find coliforms in more than 5% of the samples collected each month (US EPA, 1989). The number of samples from the distribution system that public water systems are required to take each month is proportional to the population served by the public water system and is detailed in Table 1. A Revised Total Coliform Rule was created in February 2013 to improve upon the 1989 rule, but public water systems are not required to adopt these revisions until April 1$^{st}$, 2016 (US EPA, 2013).

Table 1: Total coliform sampling frequencies required by the US EPA (US EPA, 1989)

| Population served | Public water system total coliform sampling frequency as required by the EPA |
|---|---|
| <1,000 | Once a month or less frequently |
| 50,000 | 60 times per month |
| 2,500,000 | At least 420 times per month |

In the distribution system, microbial growth and infiltration are possible. The microorganisms leaving a treatment facility might interact with microorganisms already present in the distribution system to create biofilms, which are assemblages of microorganisms that are attached to a solid surface and encased in a matrix of extracellular polymeric substances. These biofilms are microenvironments that allow for microbial growth and protection from the surrounding environment where disinfection residuals could otherwise inactivate viable cells. Microorganisms in distribution systems can corrode the pipe network through biocorrosion. Biocorrosion, along with natural pipe aging processes, lead to areas along the distribution network that allow further infiltration into municipal drinking water supply (Ingerson-Mahar et al., 2012). By the time treated drinking water reaches end users (taps), it can contain a diverse microbial community. Many studies have characterized the microbial communities present in tap water at residential taps (Henne et al., 2012), municipal drinking water (Holinger et al., 2013), swimming pools (Rose et al., 1998), hot tubs (Kahana et al., 1997), and hospitals (Anaissie et al., 2002). However, few studies have investigated the microbial communities present in residential showers.

## 2.2 Factors Affecting Microbial Diversity in Drinking Water

Many studies have examined the treatment-to-tap factors that could affect the microbial populations present in tap water. Hwang et al. (2012) studied a groundwater treatment plant that switched from chlorine to chloramine disinfection and found that the core microbial community shifted from an abundance of *Cyanobacteria*, *Methylobacteriaceae, Sphingomonadaceae,* and *Xanthomonadaceae* in chlorinated water

to *Methylophilaceae*, *Methylococcaceae*, and *Pseudomonadaceae* in chloraminated water. A study by Gomez-Alvarez et al. (2012) also found changes in microbial communities in distribution systems as a function of disinfectant residual type with more abundant *Legionella*-like genes in chlorinated drinking water and more abundant mycobacterial genes in chloraminated drinking water.

The piping material found in the distribution system is another factor that has been studied regarding its effect on the microbial communities present in drinking water. A report by Ingerson-Mahar et al. (2012) speculated that some piping materials might support the growth of certain microorganisms in distribution lines by releasing bio-available chemicals into the water. However, Ingerson-Mahal et al. (2012) also hypothesized that other materials, such as copper, could inhibit microbial growth. Consistent with these speculations, a study by Wang et al. (2012) investigated the microbial communities present in a simulated distribution system using three different piping materials - iron, cement, and PVC - and found the highest bacterial gene copy numbers (which correlate to bacterial cell numbers) when iron was used as the piping material as compared to cement and PVC. This finding can be explained as iron corrosion can interfere with disinfection, and iron rust can increase pipe roughness and surface area, thereby providing more attachment sites for microorganisms while also serving as a nutrient source (Lechevallier et al., 1993; Morton et al., 2005).

A study by Pinto et al. (2012) demonstrated that the filtration process at water treatment facilities impacts drinking water bacterial communities. This study found that the microbial populations that grow on filters in treatment processes persist in the distribution system despite disinfection and disinfectant residuals. Pinto et al. propose that altering water quality parameters to manipulate the bacterial communities that colonize filters in a treatment facility might be a suitable means to beneficially control microbial communities in distribution systems.

Taken overall, the literature identifies several factors that affect the microbial community of tap water. It is expected that these factors might also affect the microbial communities present in residential showers.

## 2.3 Microorganisms in Residential Showers

The microbial community in the shower environment is of interest in this study. Showers offer ingestion, inhalation, and skin contact risks for occupants from the microorganisms present in this environment. Several studies have examined the risk of microbial exposure from showers in hospitals and how they might relate to nosocomial infections (Anaissie et al., 2002; Angenent 2005; Perkins et al., 2009; Williams et al., 2013). Hospital showers have received special attention as they can be used for bathing immunocompromised individuals who are at a high risk for infection. However, immunocompromised individuals are not exclusive to hospitals and they are just as likely, if not more likely, to be affected by microorganisms present in their home showers, which presumably are cleansed less regularly and rigorously than are hospital showers. Additionally, studying the risk factors of microbial exposure for immunocompetent individuals in their home showers is an important task as individuals residing in the United States shower on average 0.98 times per day (Wilkes et al., 2005).

Microorganisms can be present in a variety of locations in home showers including in first flush water (from premise plumbing), bulk water (from the drinking water distribution lines), showerhead biofilms, biofilms on other shower surfaces such as walls, shower curtains, floor and ceiling, and in aerosol form. Several studies have investigated various aspects of the residential shower microbiome. Feazel et al. (2009) examined the bacterial composition of shower aerosols, water, and showerhead biofilms from 45 showers across the United States. This study found that non-tuberculosis mycobacteria and other opportunistic human pathogens were enriched in showerhead biofilms as compared to the levels present in the bulk water. In addition, Feazel et al. (2009) found that the microbial constituents present in shower aerosols were more reflective of the bulk water and not of the biofilm. Perkins et al. (2009), however, found that the bacterial communities in aerosol and water samples were significantly different. Differences in bacterial communities among water, aerosol, and biofilm samples in a bathroom could be a function of the manner in which the samples were collected or of the actual community shifts between the different

types of samples. Specifically, aerosol, water, and biofilm samples are collected differently, which could have an effect on the microbial communities detected as different collection methods might preferentially retain certain microorganisms. In addition, it is possible that differences in microbial communities between water and aerosol sampling could be due to preferential partitioning of some microorganisms from the water to air. Further, it is possible that the spray from a shower could aerosolize microorganisms on shower surfaces upon impact, thereby affecting the airborne microbial communities present during a shower. Differences in microbial communities between water and biofilm samples could be explained by the microenvironment that a biofilm creates. Biofilms can protect microorganisms from a harsh environment, such as the disinfectant residual present in the bulk water. This protective environment allows for the growth of microorganisms that might otherwise not survive in the bulk water, and it is therefore understandable that the biofilm community would differ from the bulk water.

In addition to finding differences in bacterial communities based on shower sample type, Perkins et al. (2009) investigated the possibility of using a membrane-integrated showerhead to reduce the number of bacteria present in shower water and aerosols. This study found a one log reduction in cells/$m^3$ in shower aerosols and a three log reduction in cells/L in shower water using a membrane-integrated showerhead as compared to a showerhead without a filter. Results from this study indicate that efforts to filter the water exiting a showerhead can have a substantial influence on the microorganisms present in shower water and shower aerosols.

Falkinham et al. (2008) recovered a *Mycobacterium avium* isolate from water samples and showerhead biofilm samples in a woman's shower; this isolate was clonally related to the *M. avium* isolate with which the woman was infected. Feazel et al. (2009) found *M. avium* in addition to another opportunistic human pathogen, *Mycobacterium gordonae*, in residential showers in the bulk water and in showerhead biofilms. A study by Kelley et al (2004) investigated the microbial communities present on four shower curtain biofilms and found that the genera with the highest abundance were *Sphingomonas* and *Methylobacterium*; both genera are known to include opportunistic pathogens. In addition,

Chang and Hung (2012) found *Legionella pneumophila* in shower aerosols and water samples from the shower rooms of two nursing homes. Similarly, Makin and Hart (1990) found *L. pneumophila* and *Legionella bozemanii* in showerheads and premise plumbing. These studies all have reported the presence of bacterial pathogens in the shower environment highlighting the public health relevance of characterizing bacterial communities in residential showers. However, bacteria are not the only microorganisms of concern.

Other studies have investigated the presence of fungi in tap water and showers. Fungi in tap water are important to consider as fungal infections are difficult to treat and are often fatal (Kanzler et al., 2007). Hamada et al (2010) studied the growth of fungal species commonly found in bathrooms in Japan. They found that *Ramichloridium strelitziae, Cyphellophora laciniata, Phoma fimeti*, and *Exophiala* sp. can grow on surfactants (soap and shampoo), which is likely why they are prevalent in bathrooms. Rinsing away residual surfactants and trying to keep bathroom surfaces dry were the recommended efforts to reduce fungi in bathrooms.

Hageskal et al. (2009) noted that fungi are more commonly recovered from cold water and shower water than from hot water and other tap water sources, and that air concentrations of fungi have been found to increase in areas where showers were run often, indicating an association between fungal aerosols and water. In addition, studies have shown links between fungi in tap water and reports of hypersensitivity pneumonitis, a disease in which the lungs become inflamed from inhalation of antigens such as mold, dust, and chemicals (Girard et al., 2009). Specifically, Metzger et al. (1976) reported that the fungus *Aureobasidium pullulans* in water from a home sauna was likely causing hypersensitivity pneumonitis in sauna-users, and Muittari et al. (1980) indicated that fungi such as *Aspergillus fumigatus, Mucor* spp., *Absidia* spp., and *Candida* spp. were causing symptoms of hypersensitivity pneumonitis after sauna, bathing, and shower events.

Although previous studies have shown that fungi and bacteria are present in residential shower environments, no studies to date have investigated the aerosolization of fungi in residential showers. The present study aims to address this gap in the literature by

studying the aerosolization of both bacteria and fungi during shower operation. In addition, this study attempts to isolate the aerosolization of microorganisms from tap water from those aerosolized from shower biofilms by investigating shower aerosols generated from a controlled experimental shower setup void of biofilm growth.

## 2.4 Microbial Aerosols

To assess the risk of human exposure to microorganisms present in showers, it is important to understand the fundamental characteristics of microbial aerosols. For instance, Marthi et al (1990) investigated the mortality of *Enterobacter cloacae, Erwinia herbicola, Klebsiella planticola,* and *Pseudomonas syringae* after aerosolization and found that, in general, more cells remained viable when they were aerosolized in an environment with high relative humidity (70-80%) and low temperature (12˚C). Spraying a larger droplet size (median diameter, 450 µm), also enhanced the viability of the aerosolized organisms relative to spraying a smaller droplet (median diameter, 150 µm). These results suggest that larger droplet sizes and high relative humidity conditions within shower environments might enhance the viability of aerosolized bacteria. However, the survivability of aerosolized microorganisms is a function of the type of microorganism, and thus it is difficult to broadly generalize across different microbial species.

Characteristics of specific microorganisms might result in preferential aerosolization from solution. For example, it is known that *M. avium* preferentially aerosolizes from water (Angenent et al., 2005; Falkinham, 2003; Feazel et al., 2009). Characteristics of this bacterium that likely cause its preferential aerosolization are its hydrophobic character and ability to concentrate on gas bubbles (de los Reyes and Raskin 2002). In addition, the shower environment in general could increase the potential for aerosol partitioning of any microorganism with a hydrophobic cell wall as high temperatures and the presence of surfactants can greatly reduce aerosol/water interface surface tension (Angenent et al., 2005).

Aerosol-water partition coefficients that can be used to estimate the distribution of microorganisms between water and air have been developed for a few species. For instance,

Schoen and Ashbolt (2011) used a partition coefficient for *Legionella* to develop a model to predict the concentration of *Legionella* in shower water, biofilms, and air that would result in pulmonary infection. This aerosol-partition coefficient was calculated as the average concentration of bacteria in the air divided by the concentration in the source water. It was determined that, with moderate uncertainty, the amount of *Legionella* required in the shower environment to produce an air concentration resulting in infection is within the range of the levels that have been measured in residences (Schoen and Ashbolt, 2011). Development of aerosol-water partition coefficients for other microbial species following the framework presented by Schoen and Ashbolt (2011) would be a beneficial addition to the shower microbiome literature as this would allow for estimates of the concentration of microorganisms in shower aerosols given their concentration in water.

Fungal aerosols in bathrooms come from outdoor sources as well as indoor and water sources. Fungal spores present in water sources might preferentially aerosolize as they tend to be hydrophobic in water (Reponen et al., 1996). Additionally, inhalation of intact fungal spores is not the only source of concern with respect to human health because fungal fragments also can cause adverse health effects. Fungal fragments are smaller than fungal spores allowing them to travel deeper into the respiratory system, and they can deliver harmful antigens and mycotoxins to the alveolar region of the lung (Cho et al., 2005).

## 2.5 Microorganisms of Interest

Based on previous studies (Bush et al., 2004; Falkinham et al., 2001; Feazel et al., 2009) and preliminary shower aerosolization studies (Kinney, 2012) a bacterial complex and a fungal species, *M. avium* complex and *A. alternata,* respectively, were chosen as target organisms for this study. Both of these microorganisms are known to be found in residential tap water, and they both pose a health risk to humans.

*M. avium* complex has been discussed widely in the literature as an important opportunistic pathogen found in tap water. *M. avium* complex is composed of two different species of *Mycobacterium*: *M. avium* and *Mycobacterium intracellulare* (Falkinham et al.,

2001). These organisms are unique in that they can grow in water over a range of temperatures, from 15˚C to 45 ˚C (George et al., 1980) and are resistant to chlorine-based and ultraviolet disinfection (Taylor et al., 2000). In addition, *M. avium* complex can grow in biofilms adding further resistance to disinfection (Falkinham et al., 2001). Due to *M. avium* complex's resistance to disinfection, water treatment methods and home cleaning methods that rely on chlorine-based detergents might select for this microorganism and enhance its proliferation in distribution systems and home surface biofilms. *M. avium* complex is very persistent, and in one study it was shown to survive for as long as 41 months in a distribution system (von Reyn et al., 1994). Likely because of this organism's persistence, no strong seasonal effect on its presence in water distribution systems has been observed (Falkinham et al., 2001). Another unique characteristic of *M. avium* complex is its ability to engage in parasitism in free-living protozoa, specifically amoeba, in potable water (Salah et al., 2009). This is another means of resilience for this bacterium because parasitism provides a protective microenvironment.

As noted earlier, *Mycobacterium avium* preferentially aerosolizes. There has been an increase in shower usage over baths in past decades, and it is believed that this has been a cause for the increase in non-tuberculosis mycobacterium infections because showers provide a niche for mycobacterial biofilm growth and subsequent aerosolization (Whiley et al., 2012). *M. avium* complex is the most common cause of non-tuberculosis mycobacterium infections in developed countries (Whiley et al., 2012).

With respect to health effects caused by this opportunistic pathogen, *M. avium* complex has been found to cause pulmonary infections in individuals with preexisting lung conditions and has been recovered from elderly women without known risk factors for *M. avium* complex infection (Prince et al, 1989; Wolinsky et al., 1979). In addition, *M. avium* complex has been found to cause hypersensitivity pneumonitis (Marras et al., 2005) and gastrointestinal tract and disseminated infection in immunocompromised individuals (Nightingale et al., 1992). Because of its prevalence and medical importance, *M. avium* complex was chosen as the bacterial microorganism to examine in this study.

In addition to choosing a complex of bacterial pathogens as microorganisms of interest in this study, it was deemed important to choose an allergenic fungal species of interest as fungi are common water and air contaminants, and because their occurrence in drinking water has been understudied. *A. alternata* was chosen as the fungal species of interest in this study as it is a known allergen and one of the most common fungi associated with asthma (Bush et al., 2004). In addition, in a preliminary shower aerosolization study (Kinney, 2012), *A. alternata* was the most abundant fungal bioaerosol recovered and its relative abundance increased in samples collected during shower operation. Further, Salo et al. (2006) found that exposure to *A. alternata* in homes in the United States is associated with active asthma symptoms. Babiceanu et al. (2013) exposed human airway epithelial cells to *A. alternata* spores in vitro and determined that *A. alternata* proteins and/or metabolites likely act as inducers of epithelial inflammatory responses.

*A. alternata* is capable of growing in warm temperatures, at least up to 37˚C (Oliveira et al., 2013), and can enter the indoor environment both through municipal water sources as well as outdoor sources. Although there is significant seasonable variability in outdoor air concentrations of *A. alternata*, with higher concentrations during summer and fall months when atmospheric spore counts peak (Bush et al., 2004), indoor concentrations of *A. alternata* do not reflect these seasonal patterns implying that outside penetration is not the dominant mechanism for *A. alternata* presence indoors (Salo et al., 2006). In addition to human health issues, *A. alternata* in particular, and waterborne fungi in general, are associated with taste and odor problems (Bays et al, 1970).

Kanzler et al. (2008) investigated the presence of fungi in thirty-eight drinking water and groundwater sources in Austria; fungi were found in every sample, and *A. alternata* was found in 2.6% of all water samples. *A. alternata* also was found in samples from municipal water supply mains in the United Kingdom in a study by Kinsey et al. (1999). Along with their presence in municipal bulk water, a study by Doggett (2000) found *A. alternata* in biofilms within a municipal water distribution system in Kansas. Because of their abundance in the drinking water distribution systems and indoor air, as well as the lack of information currently available in the literature regarding *A. alternata*

in the shower environment, this organism was chosen as the fungal species of interest in this research.

## 2.6 Physical Characterization of Residential Showers

Particles that are emitted during a shower can span sizes of less than 0.3 microns to greater than 10 microns in diameter (Xu and Weisel, 2003). Small particles in the 1- to 5-micron diameter size range deposit in the alveoli of humans (Thomas et al., 2008). Many bacterial cells and fungal spore fragments fall into this size range including, for example, species of the common indoor fungal genera *Aspergillus, Cladosporium,* and *Penicillium* (Reponen et al., 1996) and bacterial genera *Staphylococcus, Bacillus,* and *Aeromonas* (Górny et al., 1999). Thus, it is possible that the microorganisms present in shower aerosols can travel deep into our respiratory tract. Larger particles, greater than 10 microns, deposit in our upper respiratory tract (Thomas et al., 2008). This size range is consistent with larger microbial cells and spores and aggregates of microorganisms (e.g., pieces of dislodged biofilm aerosolized from a showerhead or shower surface). In addition to the inhalation risk of shower microorganisms, there is also a risk for ingestion and skin contact. Physical characteristics of showers that might affect the delivery of shower microorganisms to our skin, respiratory tract, and mouth include air exchange rate, relative humidity, water temperature, flow rate, spray pattern of the showerhead, position of the showerhead relative to the occupant, among other factors.

Few studies have sought to characterize these physical attributes of showers, and the research presented in this thesis resolves to fill in some of these gaps in the literature. A study by Chen et al. (2003) looked at the effect of two different showerheads (jet-flow and spray type) on the emissions of volatile organic compounds (VOCs) from shower water to air. The results indicate that an increase in the number of nozzles on a showerhead produces more water droplets during shower operation, providing a larger water droplet surface area and therefore increased potential for mass transfer (Chen et al., 2003). In addition, this study created a model to predict VOC emissions during showering as a factor of a variety of shower characteristics. Simulations of this model predicted that a lower

water temperature, lower showerhead height, an increase in air exchange rate and an increase in the shower-stall volume should result in reduced VOC exposure risk during showering (Chen et al., 2003). Other studies reported results consistent with Chen et al. (2003) and found that a lower shower water temperature produces fewer particles (Cowen and Ollison, 2006; Zhou et al., 2007). However, Keating et al. (1997) investigated bathroom chloroform concentrations during shower operation with different showerheads and found that the showerhead generating fewer, larger droplets resulted in the highest bathroom chloroform concentrations, contradictory to results from Chen et al. (2003). This result was explained, however, by the possibility that the wider spray pattern of the showerhead producing larger droplets allowed these particles a longer air residence time than the showerhead producing smaller particles with a more compact spray pattern. This longer residence time allowed for greater mass transfer of chloroform from the water to the air phase.

Mechanisms for shower particulate removal are of importance, and have been discussed in a limited number of publications. While the shower is running, it is speculated that diffusion and convection related to air exchange are the main factors responsible for the removal of small particles, less than 2 microns in diameter (Owen et al., 1992). However, when the shower is turned off, one study suggested that evaporation is the dominant mechanism for small particle removal as the humidity in the shower is decreasing (Xu and Weisel, 2003). The same study suggested that the removal mechanisms for particles larger than 2 microns, both when the shower is running and when it is turned off, are likely a combination of gravitational settling, thermophoresis (or the movement of particles in response to a temperature gradient), and evaporation (with evaporation playing a larger role when the shower is turned off) (Xu and Weisel, 2003). In addition, Xu and Weisel, (2003) found that particles less than 0.3 microns in diameter make up the majority of the particles present during shower operation.

Of the two studies that investigated the effects of air exchange rate on shower aerosols, neither study controlled air exchange rates in their experiments; Chen et al. (2003) used a model to simulate different air exchange rates, and Cowen and Ollison (2006) made

their conclusions regarding air exchange rate based on the small variance in the air exchange rate observed in the residential shower they studied. The research presented in this study uses an experimental shower set up in a chamber to control air exchange rate and to examine the effect of air exchange rate on the relative size of the aerosols produced. To the author's knowledge, this is the first study to measure the effect of air exchange rates on particle emissions and relative humidity in a shower.

## 2.7 Fungal Illumina® Sequencing Considerations

This study utilized high-throughput Illumina® MiSeq sequencing to identify the dominant bacterial/archaeal and fungal populations present in shower samples collected from residences and an experimental shower set-up. The use of Illumina® sequencing for delineating bacterial/archaeal communities based on regions of the 16S rRNA gene has become an accepted method (Claesson et al., 2010; Lazarevic et al., 2009), but this method of analyses for fungal communities is still under development. The currently accepted method for fungal sequencing remains pyrosequencing, as this platform provides longer sequences (400-600 bp) than does Illumina®. These longer sequences have been presumed to be necessary for accurate identification of fungi (Bokulich & Mills, 2013). However, the Illumina® platform provides better coverage than pyrosequencing, allowing a higher sequencing capacity per run and a significantly lower cost per sample making this method increasingly popular. Bokulich & Mills (2013) demonstrated the utility of the Illumina® sequencing platform for the study of fungal communities for several internal transcribed spacer (ITS) primer sets. Although Illumina® yields shorter sequences than does pyrosequencing, it appears that the sequence lengths are sufficient for genus-level taxonomic classification of ITS sequences. Nevertheless, all sequencing techniques can include bias, and those biases affecting Illumina® sequencing of fungal communities must be addressed. Biases are introduced to Illumina® sequencing methods based on several factors including which ITS sub-region is chosen for PCR amplification prior to a sequencing run and the computational method chosen for taxonomic classification.

Previous studies have shown that the PCR amplification of the ITS1 sub-region of the fungal-conserved ITS region provides more reliable community reconstruction than does the ITS2 sub-region or whole ITS region (Bokulich & Mills, 2013; Bellemain et al., 2010). This is likely due to (1) the shorter amplicon length providing better coverage of ultravariable DNA loci and (2) the distribution of the ITS1 sub-region, which minimizes preferential amplification biases of certain groups of fungi (Bokulich & Mills, 2013; Bellemain et al., 2010). Further, Bokulich & Mills (2013) found that Illumina® sequence data that used ITS1 primers exhibited genus-level accuracies of >95%, thereby demonstrating the utility of this sequencing platform for genus-level sequence reads. In addition to biases introduced by the targeted amplification region of the ITS region, the computational method used for taxonomic classification also generates bias. Porter and Golding (2011) compared two similarity-based methods (BLAST and MEGAN) and one phylogeny-based method (Statistical Assignment Program) for the taxonomic classification of high-throughput sequence reads. They found that BLAST has the highest recovery rates and MEGAN has the lowest erroneous recovery rates across partial and full-length ITS sequences. As BLAST has the highest recovery rate and a low erroneous recovery rate (although not as low as MEGAN), this method of taxonomic classification was chosen for the current study.

The Illumina® sequencing methods used in the present study sought to minimize the biases identified in other studies by utilizing PCR amplification of the ITS1 sub-region of the ITS region and a BLAST-based method for classification of Illumina®sequence reads. Although biases are still present in the form of primer bias/mismatch, amplicon length bias, and computational bias, the dominant fungal communities present are likely adequately profiled (Bokulich & Mills, 2013), which suits the objectives of the fungal sequencing for the present study.

# CHAPTER 3. MATERIALS AND METHODS

## 3.1 Recirculating Experimental Shower

### CHAMBER DIMENSIONS AND AIR EXCHANGE RATE

The chamber used to house the recirculating experimental shower unit was located at the Center for Energy and Environmental Resources at the University of Texas at Austin Pickle Research Center. The chamber had dimensions of 2.44 m long × 1.83 m wide × 2.44 m high and a total volume of 10.87 $m^3$, which is consistent with the size of bathrooms utilized in previous studies (Cowen and Ollison, 2006; Zhou et al., 2007). Air exchange rate was controlled by adjusting two variable voltage regulators (Model No. SC-3M, ISE Inc., Cleveland, OH) connected to two fans responsible for controlling the flow rate at which air was moved into and out of the chamber. A GTx116 Digital Transmitter and linear airflow sensor (Ebtron Inc., Loris, SC) were used to measure the linear flow rate of air through the chamber. The volumetric flow rate (Q) of air needed to achieve a desired air exchange rate was calculated using Equation 1:

$$Q\ \left[\frac{m^3}{hr}\right] = q * 113.4 = AER\ [hr^{-1}] \times bathroom\ volume\ [m^3] \qquad \text{(Eq. 1)}$$

Where q is the linear flow rate output from the Digital Transmitter, and 113.4 is the conversion factor necessary to turn this instrument's linear flow rate output into a volumetric flow rate. The variable voltage regulators were adjusted until this desired linear flow rate output was achieved.

The air entering the chamber was filtered using a High Efficiency Particulate Air (HEPA) filter to remove particulates, including microorganisms, larger than 0.3 microns in diameter. A DG-500 Pressure Gauge (The Energy Conservatory, Minneapolis, MN) was used to ensure that the pressure inside the chamber remained positive minimizing the infiltration of unfiltered air from outside of the chamber.

## EXPERIMENTAL SHOWER CONSTRUCTION

A recirculating experimental shower was constructed for use in the chamber studies. A schematic of the recirculating shower is shown in Figure 1. In this shower, a 27-gallon polypropylene container (Sterilite Corporation, Townsend, MA) was used as the shower basin. This basin drained into a 19-gallon polypropylene container (Sterilite Corporation, Townsend, MA), which was used as a reservoir to hold the recirculating water. PVC piping was used as support rods to hold the plastic sheeting that simulated a shower curtain. Polyethylene tubing was used to connect a Flotec 4/10 horsepower submersible waterfall/utility pump (Flotec Water, Delavan, WI) to a Delta Economy 1.6 gpm water-saving showerhead (Delta Faucet Company, Indianapolis, IN). The showerhead operated with a non-variable spray pattern in which water was sprayed from four nozzles. The recirculating shower was placed inside of a 1.22-meter diameter circular rigid-plastic swimming pool to collect any water spilled or leaked during the duration of each experiment. The power cord to the recirculating pump was fed to an outlet outside of the chamber so that the shower could be turned on and off without having to enter the chamber.

The showerhead used in this study was rated for a flow rate of 6.06 L/min but only achieved a maximum flow rate of 3.44 L/min in these experiments. The reason for the lower flow rate is likely the power of the pump used. Several pumps were tested during the construction of the recirculating shower, and although the pump used did not provide the highest flow rate, it provided the best balance between achieving the desired flow rate and preventing the water temperature from going too high during operation of the recirculating shower.

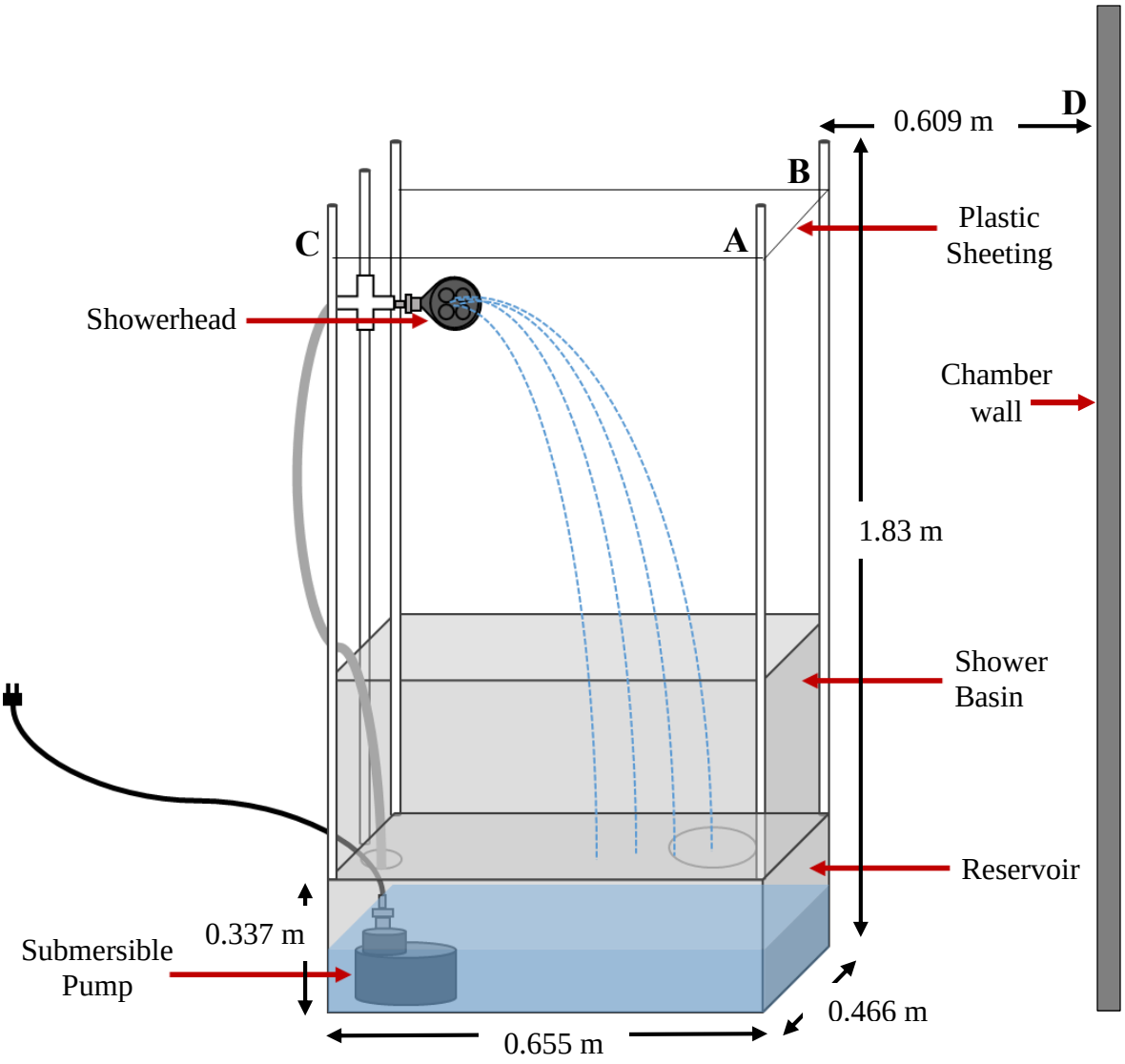


Figure 1: Recirculating experimental shower schematic. A) Location of the BioSampler during bioaerosol collection. B) Location of the Button Aerosol Sampler during bioaerosol collection. C) Location of the TSI Aerotrak particle distribution logging device. D) Location of the HOBO relative humidity and temperature data logging device

## CHAMBER OPERATION

Before each experiment, all metal surfaces in the chamber were cleaned with a sterilized 1% Contrex Powdered Labware Detergent (Decon Labs, Inc., King of Prussia, PA) solution and sterilized cloth followed by sterilized Millipore water and a separate sterilized cloth to remove any contaminants present in the chamber. Plastic surfaces were

cleaned with a 10% bleach solution and a sterilized rag followed by sterilized Millipore water and a sterilized rag. 10% bleach has proven bactericidal properties (Rutala and Weber, 2008) and therefore was chosen as the preferred cleaning solution for the chamber. However, bleach is corrosive to metals so all metal surfaces in the chamber were cleaned with detergent instead. After the chamber and shower were cleaned, the shower was filled with water from the nearest tap at the Pickle Research Center in Austin, TX.

After the shower reservoir was filled with water, the chamber was loaded with the necessary equipment to collect data and samples before the chamber was closed and sealed from the outside using ScotchBlue Multi-Surface Painter's Tape (3M Inc. St. Paul, MN) to prevent any air leaks. The air exchange rate was then raised to 26 $hr^{-1}$ for 1 hour to flush out airborne contaminants from the chamber. Then, the air exchange rate was lowered to the target air exchange rate desired for each experiment.

Two sets of experiments were run in this chamber: (1) Experiments investigating the microbial contribution to shower aerosols from tap water in a set-up void of biofilm growth (hereafter called Chamber Tap Water Bioaerosol Experiments) and (2) experiments investigating the effect of shower water temperature and air exchange rate on shower aerosol particle size distributions, bathroom air temperature, and bathroom relative humidity (hereafter called Chamber Physical Shower Characteristics Experiments). For the Chamber Tap Water Bioaerosol Experiments, the chamber was entered once during the experiment to remove 'before-shower' bioaerosol samples (bioaerosol samples collected before the shower was turned on). Afterwards, the chamber was again sealed and the air exchange rate was raised again to 26 $h^{-1}$ for 10 minutes to remove any particulates that might have penetrated the chamber during sample collection. The air exchange rate was then lowered to the desired experimental value before the experiment continued. For Chamber Physical Shower Characteristics Experiments the chamber remained sealed for the duration of the experiment.

After the completion of an experiment, the recirculating experimental shower was drained and the shower and pump were dried to prevent standing water that could promote microbial growth. The showerhead was removed, taken apart, and dried as well to

discourage microbial growth. Prior to subsequent experiments, the showerhead was wiped with 10% bleach before reassembly and reattachment to the recirculating experimental shower.

## CHAMBER TAP WATER BIOAEROSOL EXPERIMENTS

### Bioaerosol Sample Collection

A BioSampler (SKC Inc., Eighty Four, PA, USA) filled with 20 mL of a UV-sterilized phosphate buffered saline (PBS) and 0.01% Tween-20 (ICI Americas Inc., Wilmington, DE) solution and a Button Aerosol Sampler (SKC Inc., Eighty Four, PA, USA) fitted with a 25-mm diameter 3.0-µm pore size gelatin filter (SKC Inc., Eighty Four, PA, USA) were used to collect bioaerosol samples in the recirculating experimental shower. Four bioaerosol samples were collected during each sampling event: a simultaneous BioSampler sample and Button Aerosol Sample before the shower was turned on, and simultaneous BioSampler sample and Button Aerosol Sample during shower operation. Samples were collected for 15-45 minute durations depending on the sampling event. The BioSampler and the Button Aerosol sampler were located on the corners opposite of the showerhead in the recirculating shower (Locations A and B, respectively, in Figure 1). The BioSampler sampled at a rate of 12.5 L/min and the Button Aerosol Sampler sampled at a rate of 4 L/min. The BioSampler liquid samples were poured into a UV-sterilized 50-mL plastic centrifuge tube after collection, and the Button Aerosol Sampler gelatin filters were dissolved in 10 mL of a UV-sterilized PBS 0.01% Tween-20 solution. Both samples were stored at 4˚C immediately after collection. At the conclusion of each sampling event and within 24 hours of sample collection, the Button Aerosol samples were filtered through a 0.2-µm GTTP Isopore™ membrane filter (EMD Millipore Corporation, Billerica, MA) and the BioSampler samples were pelleted by centrifugation at 12,000×g for 5 minutes; the supernatant was discarded. Both sample types (Biosampler and Button Aerosol) were then stored at -20˚C until DNA extraction.

**Water Sample Collection**

Two water samples were collected for each sampling event: One from the tap at the Pickle Research Center directly before the recirculating shower reservoir was filled, and one from the recirculating shower reservoir at the end of the experiment. Water samples were collected in sterile 1-L glass bottles and stored at 4˚C immediately after collection. After the completion of the experiment, the water samples were filtered within 24 hours of collection through a 0.2-µm GTTP Isopore™ membrane filter and then stored at -20 ˚C until DNA extraction.

**Experimental Parameters**

Simultaneous bioaerosol samples were collected using both bioaerosol sampling devices (the BioSampler and the Button Aerosol Sampler) before and during shower operation to determine the impact of tap water on shower bioaerosols. Sampling duration (for both the before and during shower bioaerosol samplings), air exchange rate, and water temperature were varied to determine their effect on the tap water contribution to bioaerosolization. In addition, water samples were collected during each sampling event to investigate similarities between microbial communities present in shower aerosols and tap water. A summary of the Chamber Tap Water Bioaerosol Experiments are shown in Table 2 including the experimental variables (water temperature, sampling duration, and air exchange rate) as well as the water flow rate and chamber pressure for each sampling event. Each sampling event occurred on different days between June and August, 2013. The chamber and recirculating experimental shower were cleaned before and after use as previously mentioned in the chamber operation section of this study.

Table 2: Experimental parameters for the recirculating shower experiments to determine microbial contributions to shower aerosols from tap water

| Sampling event | Water temperature (˚C) | Sampling duration (min) | Air exchange rate ($hr^{-1}$) | Water flow rate (L/min) | Chamber Pressure (Pa) |
|---|---|---|---|---|---|
| 1 | 46 | 15 | 2.5 | 3.44 | 1.1 |
| 2 | 46 | 15 | 5.0 | 3.27 | 4.0 |
| 3 | 46 | 15 | 8.0 | 3.40 | 7.4 |
| 4 | 25 | 15 | 2.5 | 3.34 | 1.1 |
| 5 | 35 | 15 | 2.5 | 3.04 | 1.3 |
| 6 | 46 | 45 | 2.5 | 3.28 | 1.1 |

Sampling events 1-3 held sampling duration and water temperature constant while varying air exchange rate from 2.5-8.0 $hr^{-1}$. Sampling events 1, 4, and 5 held sampling duration and air exchange rate constant while varying water temperature. Sampling events 1 and 6 held water temperature and air exchange rate constant while increasing sampling duration from 15 to 45 minutes.

## CHAMBER PHYSICAL SHOWER CHARACTERISTICS EXPERIMENTS

### Particle Size Distribution Data Collection

Semi-continuous particle counts were recorded during each recirculating shower experiment using a TSI Aerotrak handheld particle counter (TSI Inc., Shoreview, MN, USA). Particle counts were recorded at 1-minute intervals with 15 seconds between each reading period. The size channels used were 0.3-1.0, 1.0-2.0, 2.0-3.0, 3.0-5.0, 5.0-10.0, and >10 µm. The intent was to place the TSI Aerotrak in an area near the breathing zone in a shower. However, the TSI Aerotrak could be damaged by direct water contact, so this instrument was located near the showerhead but outside of the shower (Location C in Figure 1). It is possible that this particle data collected is not entirely representative of the particles present in the breathing zone of an occupant in a shower, however this data is used for comparative purposes only, and as such it is useful.

**Temperature and Relative Humidity Data Collection**

Temperature and relative humidity were continuously measured using a HOBO Data Logger (Onset Computer Corporation, Bourne, MA, USA). Temperature and relative humidity readings were logged every five seconds for the duration of the experiment. The HOBO Data Logger was located on the wall of the chamber facing the showerhead approximately 2 feet from the shower and 7 feet above the ground (Location D in Figure 1).

**Experimental Parameters**

To determine the effect of water temperature and air exchange rate on the particles generated in the experimental shower, the particle size distribution, temperature, and relative humidity were measured for a shower run with cold water (the coldest water produced by the tap, 23-25˚C) and hot water (the hottest temperature that the pump could recirculate without overheating, 39-41˚C). These measurements were performed separately from the bioaerosol sampling described in Table 2. For each water temperature, data were collected at air exchange rates of 0, 2.5, 5, 8, and 10 $h^{-1}$. The pressure inside of the chamber at each air exchange rate, and the flow rate of water from the showerhead for each water temperature are summarized in Table 3.

Table 3: Experimental parameters for the recirculating shower experiments to determine airborne particle size, temperature, and relative humidity during shower operation as a function of air exchange rate.

| **Water temperature (˚C)** | **Water flow rate (L/min)** | **Chamber pressure (Pa)** | | | | |
|---|---|---|---|---|---|---|
| | | **0 $h^{-1}$*** | **2.5 $h^{-1}$** | **5 $h^{-1}$** | **8 $h^{-1}$** | **10 $h^{-1}$** |
| Cold (23-25) | 3.21-3.32 | -0.9 | 1.1 | 4.0 | 8.7 | 5.9 |
| Hot (39-41) | 3.27-3.44 | -0.8 | 1.3 | 4.1 | 6.0 | 6.0 |

*It was not possible to attain a positive chamber pressure with an air exchange rate of 0 $h^{-1}$ as no air was entering the chamber

## 3.2 Residential Shower Sampling

### RESIDENTIAL SHOWER LOCATIONS

Shower samples were collected between June and August 2013 from one residence in Austin, TX, and from one residence in San Antonio, TX. The cities of Austin and San Antonio were chosen for sampling due to their proximity to the University of Texas at Austin, and the fact that they represent two different municipal water sources: surface water in Austin and groundwater in San Antonio.

### BIOAEROSOL SAMPLE COLLECTION

A near-continuous liquid input wetted wall cyclone (WWC) high throughput aerosol sampling device (Texas A&M University, College Station, TX) was used to collect bioaerosol samples during residential sampling. During bioaerosol sample collection, the bathroom fan was turned off and the door to the bathroom was closed. In addition, the researchers involved in sampling procedures wore facemasks and hooded suits to minimize the microbial impact from their human microbiome. The WWC sampled air at a flow rate of 100 L/min and concentrated the airborne particles into a volume of less than 5 mL of PBS and 0.01% Tween-20. WWC bioaerosol samples were collected in the shower before, during, and after shower operation for a duration of 15 minutes each. In addition, three 15-minute WWC baseline samples were taken in a room outside of the bathroom. WWC samples were stored at 4˚C after collection. After the shower sampling event, the WWC samples were filtered through a sterile 0.22-µm polyethersulfone Water Filter (MO BIO Laboratories, Inc., Carlsbad, CA) and frozen at -20˚C until DNA extraction.

The WWC bioaerosol sampling device was chosen for these experiments as it is capable of sampling a large volume of air quickly, while concentrating the bioaerosol particles into a small volume of liquid. In addition, the WWC has been shown to achieve high microbial collection efficiencies; the average collection efficiency for single cells and clusters of *Bacillus atrophaeus* spores over the size range of 1.2-8.3 µm has been demonstrated to reach 86% (McFarland et al., 2010).

### WATER SAMPLE COLLECTION

One composite water sample was taken from each residential shower. A sterile 1-L glass bottle with a sterile funnel was placed in the shower tub before the shower was turned on. The bottle remained in the shower for the entirety of the 15-minute shower operation at which point it was removed and immediately stored at 4˚C. This method of water sampling was used to ensure the collection of a water sample during shower operation without a human presence in the bathroom (as the human microbiome might have affected the microbial communities captured). However, it is possible that this sampling method could have introduced splashed water into the composite water sample from surfaces containing biofilms, and this should be considered when interpreting the results presented herein. After completion of the shower sampling event, the water samples were filtered through a sterile 0.22-µm polyethersulfone Water Filter (MO BIO Laboratories, Inc., Carlsbad, CA) and then stored at -20 ˚C until DNA extraction.

### BIOFILM SAMPLE COLLECTION

Sterile nylon foam swabs wetted in a sterile PBS and 0.01% Tween-20 solution were used to wipe shower surfaces where biofilms were likely to grow. One square foot areas of the shower floor, ceiling, and one wall were swabbed for a 2-minute duration. The outside of the showerhead around the nozzles and the inside of the pipe that connected to the showerhead (after removing the showerhead) were also swabbed for a 2-minute duration. All of the swab samples were collected approximately 20 minutes after the shower had operated for a 15-minute duration. After sample collection, the swabs were stored at 4˚C, tip down, in 1 mL of sterile PBS and 0.01% Tween-20. After each shower sampling event, the swabs were vortexed in their liquid at which point the swab was removed (but not discarded) and the liquid was filtered through sterile 0.22-µm polyethersulfone Water Filters (MO BIO Laboratories, Inc., Carlsbad, CA). The reserved swab and 0.22-µm filter were frozen at -20˚C until they were used together for DNA extraction.

For one of the shower sample sites in Austin, the showerhead was removed and sonicated (Bransonic Ultrasonic Cleaner) (Branson Ultrasonics Corporation, Danbury, CT)

in 1 L of PBS and 0.01% Tween-20 for 30 minutes to attempt to sample the biofilm present on the inside of the showerhead. After sonication, the liquid sample was filtered through a sterile 0.22-µm polyethersulfone Water Filter (MO BIO Laboratories, Inc., Carlsbad, CA) and stored at -20 ˚C until DNA extraction.

### SUMMARY OF RESIDENTIAL SAMPLES

Table 4 reports the nomenclature that will be used to represent each type of residential sample throughout this report, and a detailed description of each sample name that will be used.

Table 4: Residential sample nomenclature and descriptions to be used in the remainder of this report

| Sample Name | Description |
|---|---|
| Outside air (0) | Field blank bioaerosol sample collected in a room adjacent to the bathroom with a filter covering the WWC inlet |
| Outside air (1) | First of three sequential bioaerosol samples collected in a room adjacent to the bathroom |
| Outside air (2) | Second of three sequential bioaerosol samples collected in a room adjacent to the bathroom |
| Outside air (3) | Third of three sequential bioaerosol samples collected in a room adjacent to the bathroom |
| Baseline air | Field blank bioaerosol sample collected inside of the bathroom with a filter covering the WWC inlet |
| Before shower air | Bioaerosol sample collected inside of the shower before shower operation |
| During shower air | Bioaerosol sample collected inside of the shower during shower operation |
| After shower air | Bioaerosol sample collected inside of the shower after shower operation |
| Sonicated showerhead liquid | Liquid sample obtained by sonicating a showerhead in PBS plus 0.01% Tween-20 |
| Wall swab | Swab of the shower wall adjacent to the showerhead |
| External showerhead swab | Swab of the external surface of the showerhead |
| Internal pipe swab | Swab of the inside of the pipe that connects to the showerhead |
| Ceiling swab | Swab of the ceiling above the shower |
| Floor swab | Swab of the shower floor |

## 3.3 DNA Extraction and Quantification

For all residential shower samples and samples from sampling events 1-5 of the Chamber Tap Water Bioaerosol Experiments, DNA was extracted using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. For sampling event 6 in the Chamber Tap Water Bioaerosol Experiments DNA was extracted with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions except for a modification to add 100 µL of 3 mg/mL lysozyme and 300 µL of phenol chloroform isoamyl alcohol (24:24:1) to increase DNA yield (Doyle and Doyle, 1987; Krsek and Wellington, 1999).

DNA was quantified using the Quant-iT™ dsDNA Assay Kit, high sensitivity (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. A standard curve was made for *Escherichia coli* DNA with mass ranging from 0.25 ng – 5 ng (See Appendix, Figure A1) from which all sample DNA concentrations were calculated.

## 3.4 Sequencing and Sequence Analysis

Extracted DNA from a residential shower in Austin, TX, and from a residential shower in San Antonio, TX were sent to the Genomic Sequencing and Analysis Facility at the University of Texas at Austin (Austin, TX) for bacterial and archaeal Illumina® paired-end (2×250) sequencing on the MiSeq platform. First-round PCR was used to amplify the V4 and V5 regions of the 16S rRNA gene using the primers 515F 515F (5'-GTGYCAGCMGCCGCGGTA-3') (Baker et al., 2003) and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') (Wang & Qian, 2009). This first round of PCR amplification was run in triplicate for each sample, pooled, and then cleaned using AMPure beads (New England Biolabs, Ipswich, MA). Second-round PCR amplification was performed with different primers that added sample-specific barcodes. Both rounds of PCR amplification (a total of 30 cycles) used Taq polymerase NEB Q5 (New England Biolabs, Ipswich, MA). The final PCR products for each sample after both rounds of amplification were then size-purified by removing amplicons less than 300 bp in length using AMPure

beads (New England Biolabs, Ipswich, MA) and quantified using PicoGreen (Life Technologies, Carlsbad, CA). Samples were then normalized by amplicon mass and pooled for the Illumina® run. In addition, a random subset of samples were assessed on an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA) to ensure correct amplicon size.

Aliquots of the same DNA extracts that were sequenced for the bacterial/archaeal 16S rRNA gene were sent to the Research and Testing Laboratory (Lubbock, TX) for fungal Illumina® paired-end (2×250) sequencing on the Miseq platform. For this sequencing method 35 rounds of PCR was used to amplify the ITS1 gene region of the *Fungal* ITS gene using the primers ITS-1F (5'–CTTGGTCATTTAGAGGAAGTAA–3') (Gardes & Bruns, 1993) and ITS2 (5'–GCTGCGTTCTTCATCGATGC–3') (White et al., 1990) and to attach sample-specific barcodes 8-10 bp in length. PCR amplification was performed using Qiagen Taq polymerase (Qiagen Corporation, Valencia, CA). After the PCR amplification, samples were prepared for their Illumina® sequencing run.

Bacterial/archaeal and fungal DNA sequences were processed and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). The paired-end reads were stitched using the flash command in QIIME with the following parameters: -r 250 –f 414 –s 2 –x 0.6. Operational taxonomic units (OTUs) were grouped at 97% similarity with an open-reference algorithm in QIIME using UCLUST. The Greengenes bacterial/archaeal reference database was used for picking bacterial/archaeal OTUs, and the UNITE fungal reference database was used for picking fungal OTUs. All samples were rarefied to the number of sequences present in the sample with the least number of sequences. Rarefactions curves were checked to ensure that representative diversity in every sample was captured after rarefaction (rarefaction curves are included in the Appendix, Section 7.5). Representative diversity for samples were assumed if the rarefaction curves for a samples (or group of samples) started to level off at the number of sequences to which the sample(s) was rarefied.

QIIME was used to generate weighted and unweighted UniFrac PCoA plots using the following string of scripts: beta_diversity,py, principal_coordinates.py, and

make_2d_plots.py. Weighted UniFrac PCoA plots take into account the relative abundance of OTUs in each sample which is not done in unweighted UniFrac PCoA plots.

## 3.5 Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was conducted to determine the prevalence of *M. avium* complex and *A. alternata* in residential shower samples. All qPCR reactions were run in triplicate on an Applied Biosystems Viia™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). All samples were analyzed at a 5×, 10×, and 100× dilution to check for inhibition. The 5× dilution generally provided the highest gene copy concentrations for every sample. In samples where a higher dilution provided a larger gene copy concentration, inhibition was assumed, and the higher dilution was used to calculate that sample's gene copy concentration. Every qPCR reaction included a standard curve.

qPCR for *M. avium* complex was conducted with a method modified from Feazel et al. (2009), which amplifies a region of the 16S gene that is conserved among members of the *M. avium* complex with a length of approximately 193 bp, that is only present once in the genome of the *M. avium* subsp. *paratuberculosis* (the standard DNA used in this assay). The primers used for this assay were 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and MAV199R (5'-ACC AGA AGA CAT GCG TCT TG-3'). Each 20-µL reaction consisted of 10 µL 2×SYBR® Select Master Mix (Applied Biosystems, Foster City, CA), 1 ng/µL each primer, 3.2 µL 1×BSA, and 1.5 µL template DNA. Standard curves were generated using genomic DNA from *M. avium* subsp. *paratuberculosis* (ATCC® BAA-968D-5™). An example standard curve and calculations for gene copies/µL are included in the Appendix, Section 7.4. The *M. avium* subsp. *paratuberculosis* standard amplified in every reaction. The *M. avium* complex specific qPCR assay included an enzyme activation step of 50˚C for 2 minutes and an initial denaturation at 94˚C for 10 minutes, followed by 45 cycles of 94˚C for 15 seconds, 60˚C for 45 seconds, and 80˚C for 1 second with a subsequent fluorescence plate read. A melting curve was constructed at the end of each *M. avium* complex qPCR run to ensure specificity in each sample's amplification.

qPCR for *A. alternata* was conducted with a method modified from Yamamoto et al. (2011) that amplifies a gene region that is conserved in the *A. alternata* species, occurs once in the genome, and is approximately 100 bp in length.. The primers and probe used in this assay were AaltrF1 (5'-GGC GGG CTG GAA CC TC-3'), AaltrR1-1 (5'-GCA ATT ACA AAA GGT TTA TGT TTG TCG TA-3'), and AaltrP1 (5'-TTA CAG CCT TGC TGA ATT ATT CAC CCT TGT CTT T-3'), respectively (Haugland and Vesper, 2002). The reporter and quencher used for the probe in this assay were 6-FAM on the 5' end and TAMRA on the 3' end. Each 20-µL reaction consisted of 1 µM each primer, 0.08 µM probe, 10 µL 2×TaqMan Universial PCR Master Mix, and 1 µL template DNA. Standard curves were generated using DNA extracted (with the same method as for the environmental samples) from a liquid culture of *A. alternata* Keissler, anamorph (ATCC® 66981™). An example standard curve and calculations for gene copies/µL and gene copies/$m^3$ air (for bioaerosol samples) are included in the Appendix, Section 7.3. The *A. alternata* standard amplified in every reaction. The *A. alternata* qPCR assay included an enzyme activation step of 50˚C for 2 minutes and an initial denaturation at 95˚C for 15 minutes followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 1 minute with a subsequent fluorescent plate read.

For both the *M. avium* complex qPCR method and *A. alternata* qPCR method, the limit of detection was calculated as the lowest gene copy concentration at which 95% of the samples positively amplified. Calculations for the limit of detection for both *M. avium* complex and *A. alternata* qPCR methods used in this study are included in the Appendix (Section 7.4 and 7.3, respectively).

# CHAPTER 4. RESULTS AND DISCUSSION

Results and discussion for each experimental objective (outlined in Section 1.2) are presented in this section. First sequencing results are used to confirm the presence of genera containing the *M. avium* complex and *A. alternata* species of interest. The presence and abundance of these species were then investigated using qPCR. Second, the contribution of tap water to shower bioaerosols is investigated. Lastly, the effects of air exchange rate and water temperature on shower particle size distributions, air temperature, and relative humidity are presented.

## 4.1 Opportunistic Human Pathogens and an Allergenic Fungus in Residential Showers: Presence and Potential Sources

### MYCOBACTERIA

A complete set of shower samples from the Austin residence were sequenced for a region of the 16S rRNA gene. A taxonomic bar chart showing genus-level diversity in the 14 Austin shower samples is shown in Figure 2. The legend in Figure 2 only lists ten of the most abundant genera in the Austin shower samples as a complete list of the genera present in these samples is unnecessary for the purposes of this study. The genus-level taxonomic diversity of *Bacteria* and *Archaea* in the Austin shower samples was used to identify the presence of the *Mycobacterium* genus, which contains the opportunistic human pathogenic species of interest in this study, *M. avium* complex. As shown in Figure 2, the *Mycobacterium* genus (represented by the bright green color) is present in every sample. Figure 3 summarizes the relative abundance (%) of mycobacteria detected in the 14 Austin shower samples.

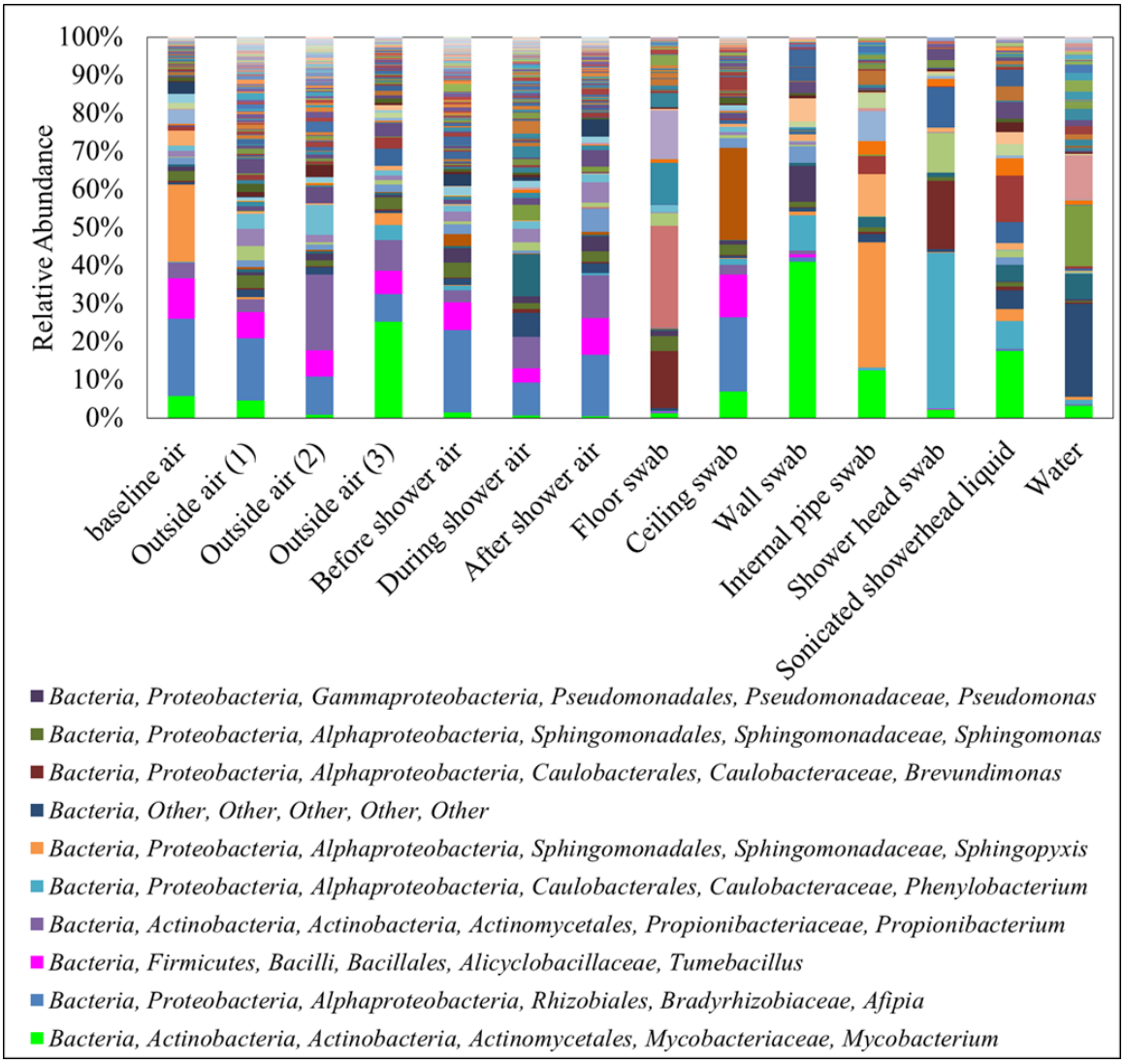


Figure 2: Bacterial and archaeal genus-level diversity in shower samples from the Austin residence. Only the ten of the genera present at the highest relative abundances are listed in the legend; over 200 genera are shown in this figure.

After establishing the occurrence of the *Mycobacterium* genus in the Austin residential shower samples, the presence of *M. avium* complex was investigated in both the Austin and San Antonio residential showers using qPCR. None of the residential shower samples from either location amplified for *M. avium* complex. This result indicates that the mycobacteria present in the Austin shower samples as indicated by the sequencing results

likely does not include the species that make up the *M. avium* complex (at least not at concentrations greater than the limit of detection of this qPCR method: 29 gene copies/µL).

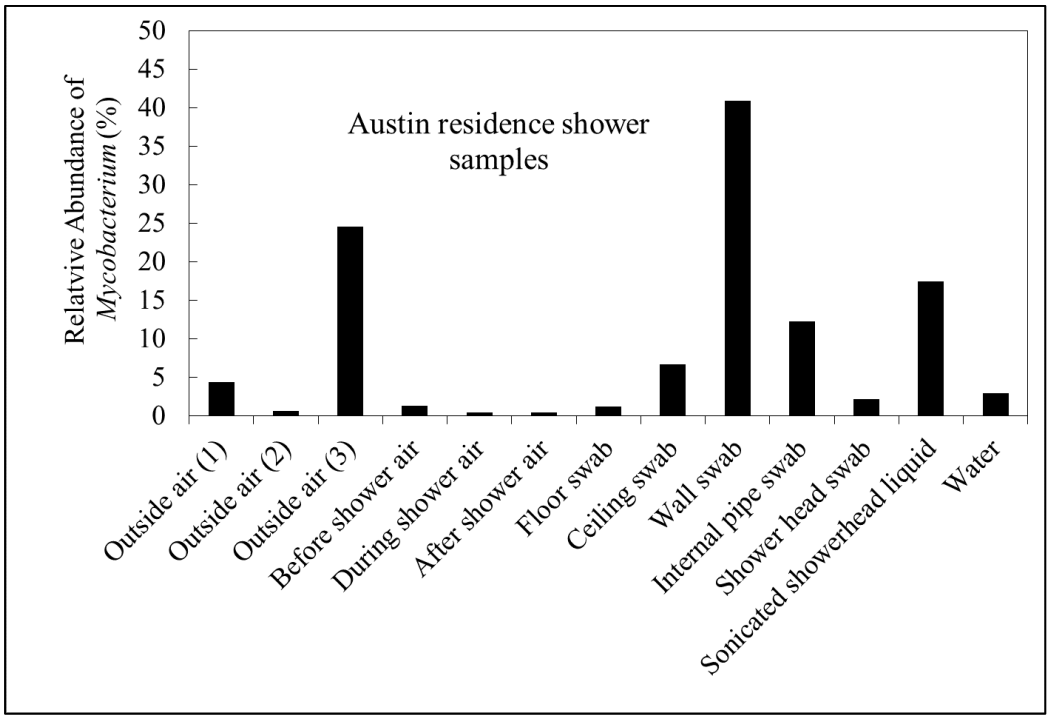


Figure 3: Relative abundance of *Mycobacterium* in a shower from Austin, TX, as indicated by sequencing data.

The *Mycobacterium* genus consists of about 100 species (Portaels, 1995), so it would not be particularly surprising if the two species that make up the *M. avium* complex were not part of the mycobacteria present in the Austin residence shower samples. Other mycobacteria that are commonly found in drinking water include the opportunistic pathogens *Mycobacterium xenopi. Mycobacterium mucogenicum, Mycobacterium kansasii, Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium gordonae,* and other non-tuberculosis bacteria such as *Mycobacterium terrae* (Chang et al., 2002; Covert et al., 1999; Falkinham et al., 2001; Vaerewijck et al., 2005; Van der Wielen and Van der Kooij, 2013). Given the abundance of opportunistic pathogens that comprise the *Mycobacterium* genus other than those that make up the *M. avium* complex, it is possible

that the mycobacteria species present in the Austin home investigated in this study could still include opportunistic human pathogens.

To determine what species would be most reasonable to target for future examination using qPCR, a random subset (20/476) of the reference sequences used by QIIME to identify the *Mycobacterium* genus in the residential shower samples were investigated for their similarity to sequences in the nucleotide collection (nr/nt) database of the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990). Reference sequences that showed a similarity of 97% or greater to a mycobacterial species from the BLASTn database are shown in Table 5 along with that species's isolate source environment.

Table 5: *Mycobacteria* species with greater than 97% similarity to a subset of reference sequences for the *Mycobacterium* genus in QIIME

| ***Mycobacterium* species** | **NCBI Accession Number** | **Isolate source environment** | **16S amplicon sequence similarity** |
|---|---|---|---|
| *Mycobacterium gordonae* | KC669528.1 | Hospital drinking water network | 99% |
| *Mycobacterium gordonae* | KC669520.1 | Hospital drinking water network | 99% |
| *Mycobacterium fortuitum* | JX119199.1 | Biofilm | 99% |
| *Mycobacterium chelonae* | JX010972.1 | Biotite mineral and soil | 99% |
| *Mycobacterium arupense* | JX575118.1 | Fish liver | 99% |
| *Mycobacterium canariasense* | KF499356.1 | Hospital tap water | 99% |
| *Mycobacterium phlei* | KF378762.1 | Activated sludge of coking wastewater | 98% |
| *Mycobacterium gordonae* | KC669525.1 | Hospital drinking water network | 98% |
| *Mycobacterium canariasense* | KF188706.1 | Hospital tap water | 98% |
| *Mycobacterium pallens* | KF378757.1 | Activated sludge of coking wastewater | 97% |

The high 16S rRNA amplicon sequence similarities between mycobacteria reference sequences from QIIME analysis of the residential shower samples and mycobacterial species sequences in the BLASTn databases do not prove that these species are present in the residences investigated in this study as the length of the reference sequences are insufficient to identify species-level taxonomy. They do, however, suggest a course for further investigation. Based on the species that were found to have high

sequence similarity to multiple reference sequences, it is recommended that qPCR be used to investigate whether *M. gordonae* and *M. canariasense* are present in residential showers in Austin and San Antonio. In addition to their high 16S amplicon sequence similarity to reference sequences, both isolates were previously found in tap water environments further supporting investigation of their presence in residential showers.

*M. gordonae*, as mentioned earlier, is another opportunistic pathogen present in the *Mycobacterium* genus. Additionally, this species has been implicated as the cause of infections in immunocompromised individuals (Panwalker & Fuhse, 1986). *M. canariasense* was discovered relatively recently, and, as such, limited data can be found in the literature regarding its pathogenicity or other potential health effects. One paper by Campos-Herrero et al. (2006), however, documents *M. canariasense* as a potential cause of catheter-related bacteremia in patients with cancer.

### *ALTERNARIA*

Samples collected from the Austin residential shower and the San Antonio residential shower were sequenced for a portion of the fungal ITS gene region. Only a subset of the samples (14 of 29) provided sequencing results, likely due to the low DNA concentrations recovered. A taxonomic bar chart showing genera-level taxonomic diversity in these samples is presented in Figure 4. Similar to the *Bacteria/Archaea* sequencing results, the legend in Figure 4 only lists the ten genera present at the highest relative abundances in the Austin and San Antonio shower samples as these data were solely used for the purpose identifying the *Alternaria* genus to justify investigation of the presence of the allergenic fungus *A. alternata*. Figure 4 shows that the *Alternaria* genus was present at a high relative abundance in many of the Austin residential shower air samples. In addition, *Alternaria* was present in many of the San Antonio air samples at lower relative abundances. Figure 5 shows the relative abundance of the *Alternaria* genus in each of the 14 shower samples collected from Austin and San Antonio that were successfully sequenced. Due to *Alternaria's* presence in many of the residential shower

aerosol samples (as determined via sequencing), the presence of *A. alternata* was investigated using qPCR.

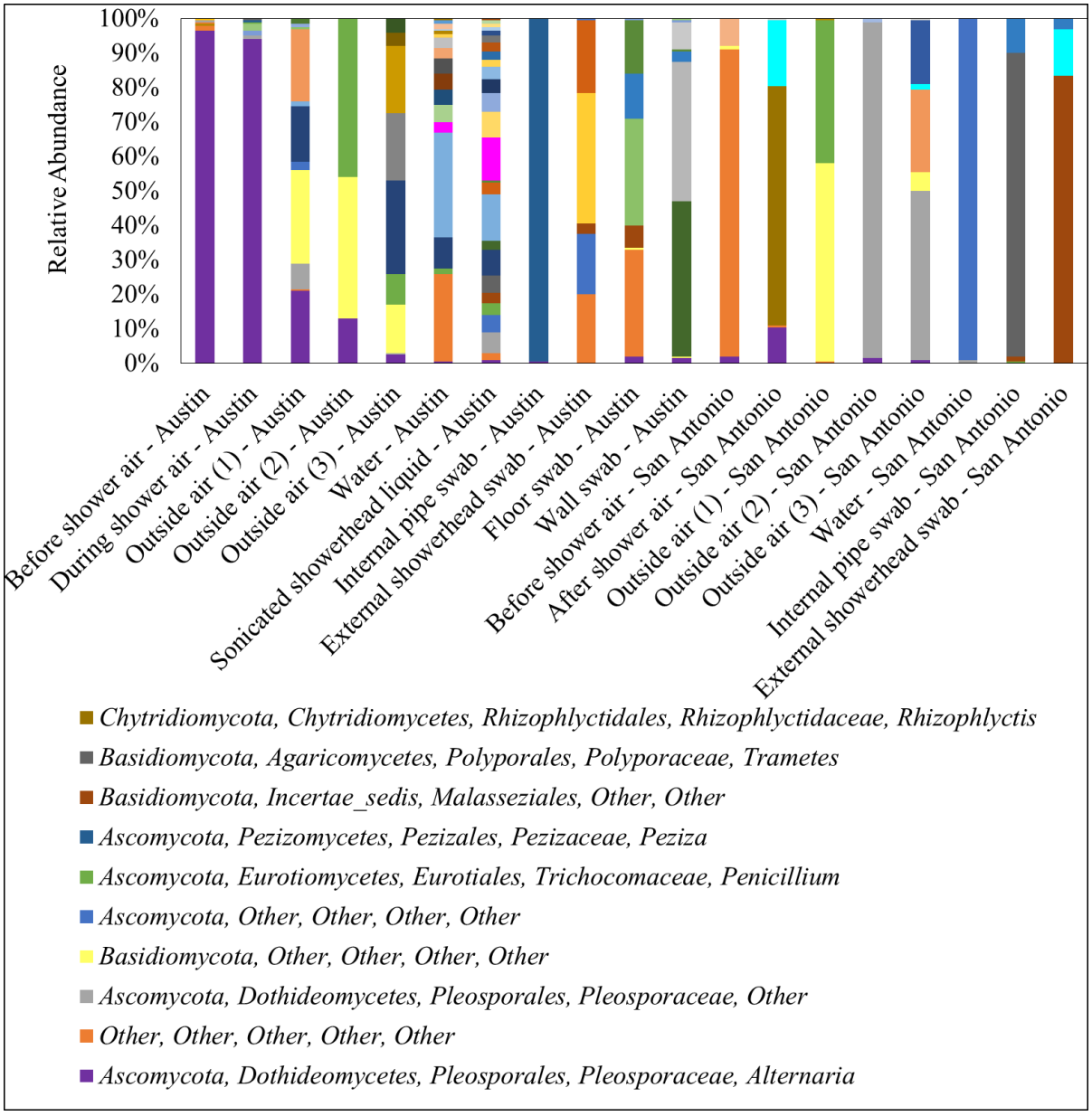


Figure 4: Fungal genus-level diversity in shower samples from the Austin residential shower and the San Antonio residential shower. Only the ten of the genera present at the highest relative abundance are listed in the legend; 71 genera are shown in the bar chart.

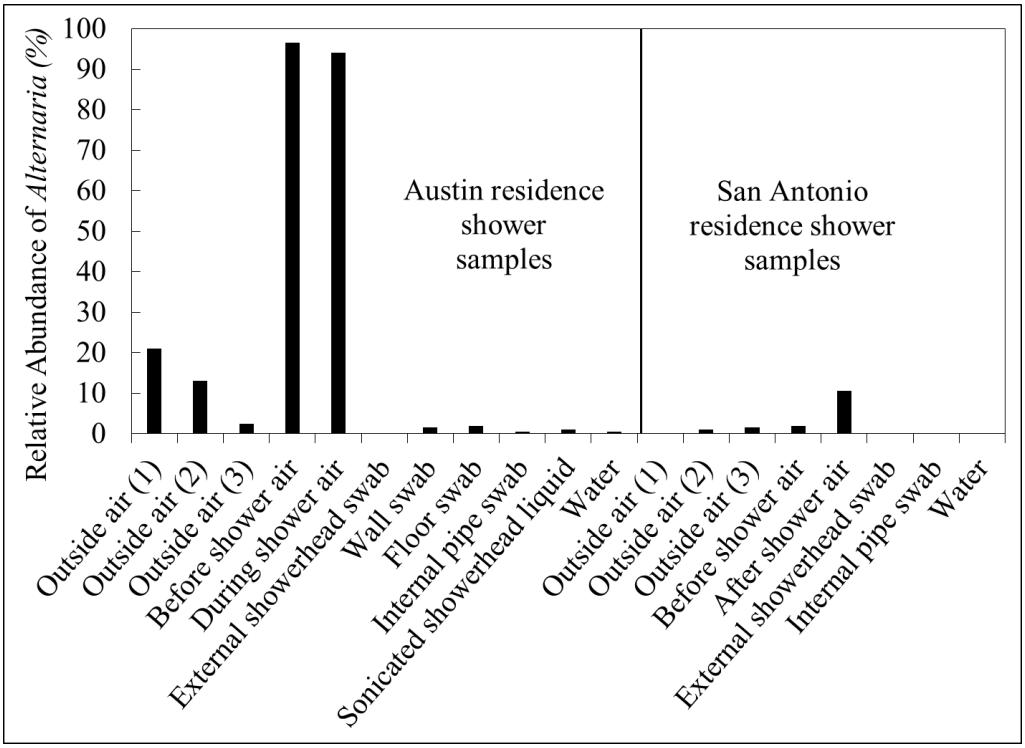


Figure 5: Relative abundance of *Alternaria* in shower samples collected from Austin, TX, and shower samples collected from San Antonio, TX, as indicated by sequencing data.

The presence and quantity of *A. alternata* was investigated using qPCR in all of the residential shower samples (both those that were successfully sequenced and those that were not). *A. alternata* was found in 9 of the 29 residential shower samples including two samples that were unsuccessfully sequenced for the fungal ITS gene: the after-shower air sample from the Austin residence and the during-shower air sample from the San Antonio residence. A description of the sample type and location as well as the number of gene copies found in each sample is provided in Table 6. The 20 samples that were tested for *A. alternata* using qPCR that did not amplify likely contained fewer amplicons (the gene

region amplified by this qPCR method) than the limit of detection of this qPCR method (2.5 gene copies/µL).

Table 6: Samples containing *A. alternata* and their respective gene copy number concentration

| Sample | Gene copies/$m^3$ air* |
|---|---|
| Before shower air - Austin | 7,532 ± 968 |
| During shower air - Austin | 93,026 ± 4,366 |
| After shower air - Austin | 46,050 ± 1,230 |
| Outside air (1) - Austin | 4,362 ± 355 |
| Outside air (2) - Austin | 912 ± 79 |
| Outside air (3) - Austin | 231 ± 70 |
| During shower air - San Antonio | 95,327 ± 3,283 |
| After shower air - San Antonio | 258 ± 103 |
| Outside air (3) - San Antonio | 193 ± 86 |

*Gene copies/$m^3$ air are reported as averages ± standard deviations of triplicate qPCR reactions.

Some samples whose sequence data supported the presence of the *Alternaria* genus did not provide a positive result for the *A. alternata* species using qPCR. This likely means that the *Alternaria* present in those samples consisted of species other than *A. alternata*. The fact that *A. alternata* was found in the after-shower air sample from the Austin residence and the during-shower air sample from the San Antonio residence that was unsuccessfully sequenced could be explained by the sensitivity of qPCR analysis. As the limit of detection for this method is 2.5 gene copies/µL, it is possible that a sample containing only a few spores of *A. alternata* could successfully amplify, making it conceivable that this method could detect the presence of microorganisms in a sample that sequencing did not. It is interesting that all of the samples that showed a relative abundance greater than 10% for *Alternaria* based on sequencing data (Figure 5) also positively amplified for *A. alternata* using qPCR. This shows that these two methods of analysis (sequencing and qPCR) appear to be in agreement.

Every sample that tested positive for *A. alternata* using qPCR was a bioaerosol sample (Table 6). The fact that no water or biofilm samples tested positive for *A. alternata* suggests that the immediate source of this fungal species in the two residential showers investigated is likely not the water. However, the highest gene copy concentrations of *A. alternata* in both residences were present during shower operation, suggesting that shower operation does impact the concentration of *A. alternata* in the shower air. It has been shown that for a different fungus (*Bremia lactucae*) an increase in relative humidity to ≥90% can drastically increase sporulation (Su et al., 2003). It is possible that an increase in relative humidity during shower operation caused sporulation of a bathroom source of *A. alternata* resulting in the increased gene copy concentration of this fungus observed via qPCR during shower operation.

*A. alternata* was only found in three samples in the San Antonio residential shower making it impossible to draw sufficient conclusions about its origin at this location. However, *A. alternata* was found in bioaerosols both inside and outside of the shower in the Austin residence, and before, during, and after the Austin residential shower operation suggesting that another source for *A. alternata* in this location could be outdoors or another source inside of the residence. *A. alternata* is a common outdoor and indoor fungus, and sampling of the two residential showers occurred in the summer when outdoor fungal counts are the highest (Bush et al., 2004). As such, it is possible that the *A. alternata* in the Austin residence could have infiltrated into the residence from outdoor sources. However, this study does not provide enough evidence to support this claim, or the claim that sporulation of *A. alternata* from a bathroom source occurred during shower operation, and further testing of outdoor air, indoor air, and additional shower surfaces at this Austin residence would be required to investigate these hypotheses.

## 4.2 Chamber Tap Water Bioaerosol Experiments

Experiments in a recirculating experimental shower setup were conducted to investigate the contribution of microorganisms in tap water to the microbial communities present in shower aerosols. Two types of bioaerosol samplers (a BioSampler and a Button

Aerosol Sampler) were used simultaneously to sample the air before and during shower operation. These samples were then sequenced for the 16S rRNA gene region conserved in *Bacteria* and *Archaea* to investigate differences in microbial communities among samples. Figure 6 shows the taxonomic diversity, after rarefication, of all of the Chamber Tap Water Bioaerosol Experiment samples. Numbers assigned to each sample indicate the sampling event during which each sample was collected (see Table 2 for sampling event parameters). Each color on this figure represents an OTU specific to the class, order,

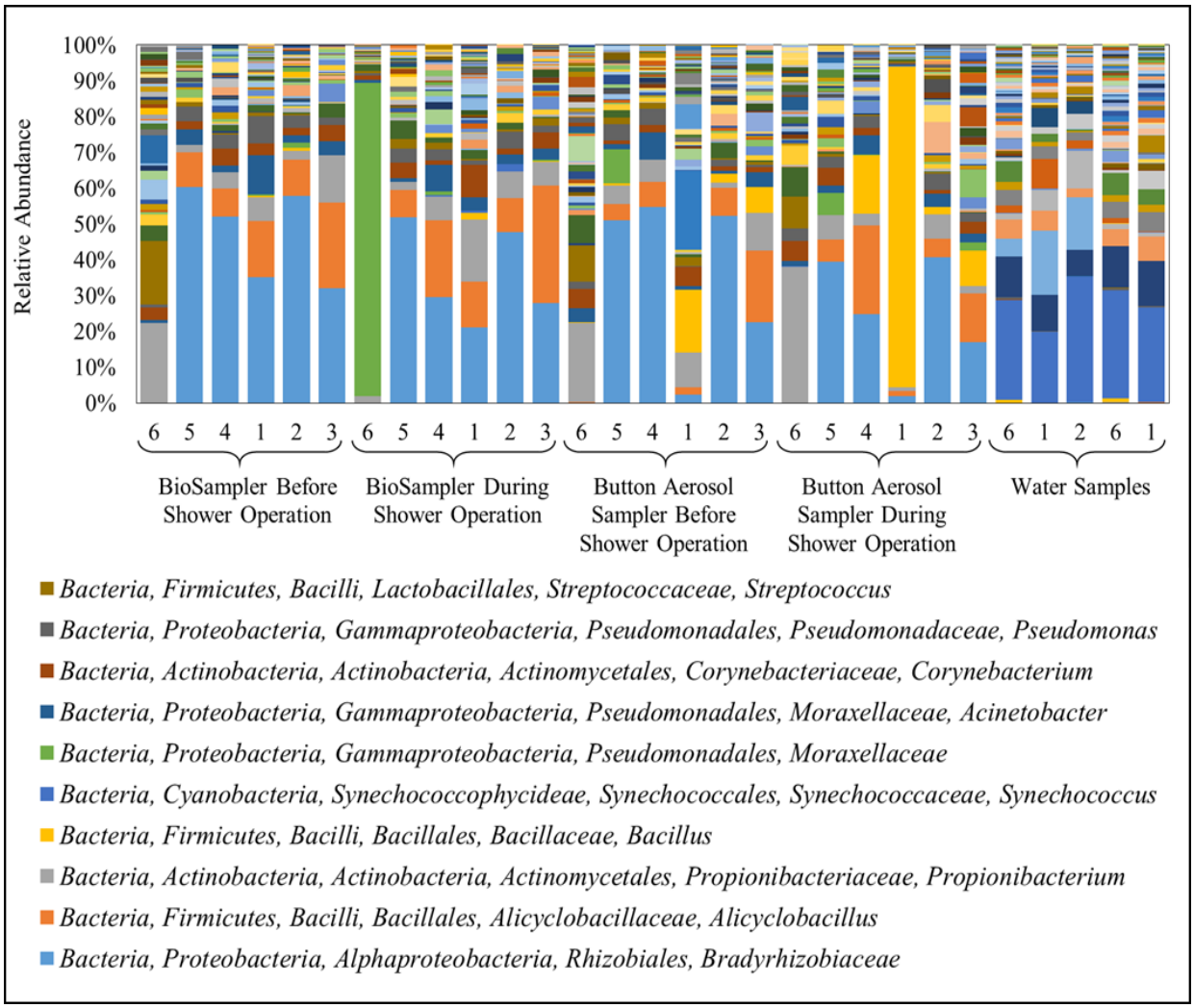


Figure 6: Bacterial and Archaeal diversity recovered from the Chamber Tap Water Bioaerosol Experiment samples.

family, or genus taxonomic classification. There are 585 distinct OTUs presented in Figure 6; however, only the most abundant OTUs in these samples are represented in the legend because a complete list is not necessary for the purpose of this investigation.

Figure 6 shows that, for each sampling event, the before-shower bioaerosols (from both bioaerosol samplers) in the chamber contain diverse bacterial/archaeal communities. This was not expected as the chamber was cleaned and flushed with HEPA-filtered air for one hour prior to each sampling event. This HEPA filter was three years old at the time of use, which might have resulted in a lower filtration efficiency and a greater background level of microbial aerosols than expected. Additionally, it is possible that the chamber flush step of raising the air exchange rate to 26 $hr^{-1}$ for one hour before each sampling event might not have sufficiently removed particles from the chamber prior to bioaerosol collection. The microbial diversity present in the before-shower bioaerosol samples makes it difficult to identify shifts in microbial communities between bioaerosol samples collected before and during shower operation.

Figure 6 also shows that the microbial communities present in the water samples differ substantially from those present in the bioaerosol samples. The *Bacteria* present at the highest relative abundance in the majority of bioaerosol samples are *Bradyrhizobiaceae* (Family), *Alicyclobacillus* (Genus), and *Propionibacterium* (Genus) while the most abundant *Bacteria* in water samples are *Synechococcus* (Genus) and *Methylobacteriaceae* (Family). *Alicyclobacillus* is a bacterium that is commonly found in soil (making its presence in aerosol samples reasonable). This bacterium can grow in a temperature range from 20-70˚C and pH values from 2.0-6.0 (Yokota et al., 2008). This bacterium is interesting as it is a poses contamination issues to the commercial beverage products industry due to its resilience at high temperature, which allows it to survive pasteurization processes (Yokota et al., 2008). *Propionibacterium* are bacteria that are commonly associated with human skin, with some species (*Propionibacterium acnes)* causing acne infections (Globa et al., 2013). *Synechococcus* is a common aquatic cyanobacterium (Scanlan & West, 2002) and, as such, its presence in water samples in this experiment is reasonable.

The distinction between water and bioaerosols in the Chamber Tap Water Bioaerosol Experiments are further exemplified in the principal coordinate (PCoA) plot shown in Figure 7. PCoA plots are used to look for sample clustering, which is indicative of phylogenetic similarity. Confidence ellipsoids are shown on each sample in both Figure 7 and Figure 8; these ellipsoids represent the variation observed for ten different randomly selected sequence subsets per sample that were used in the generation of these PCoA plots in QIIME. The water samples in Figure 7 all cluster together away from the bioaerosol samples, indicating phylogenetic dissimilarity between these two types of samples.

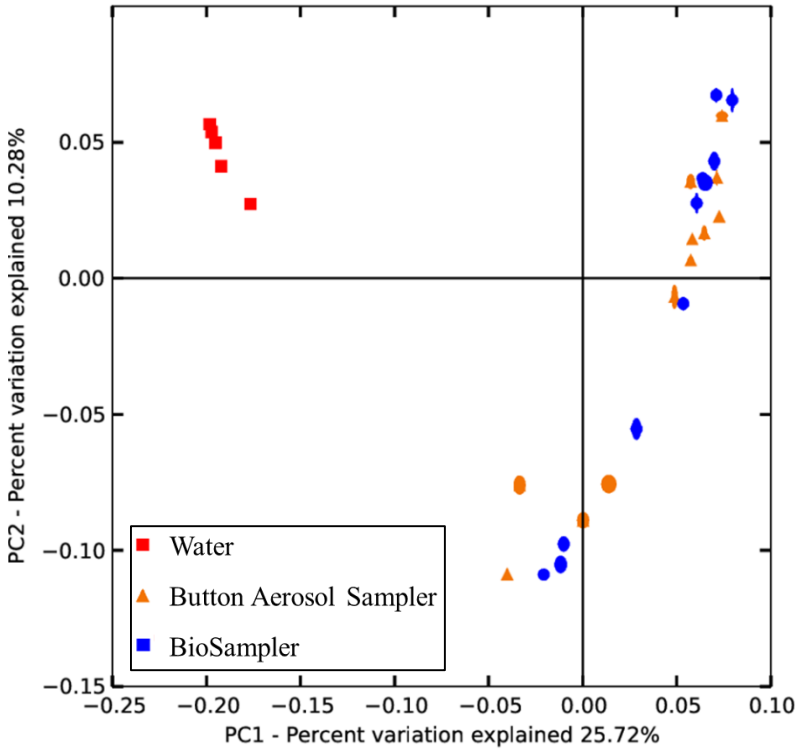


Figure 7: Unweighted UniFrac PCoA of water and bioaerosol samples from all six sampling events

Further differences between the water and bioaerosol samples are evident in the diversity present in each sample type with water samples exhibiting greater diversity. The number of distinct OTUs determined through QIIME analysis after sample rarefaction for

each of the separate bioaerosol samplers and for water are shown in Table 7. The number of unique OTUs present in the water samples are nearly double that present in either type of bioaerosol sample. A greater number OTUs in a particular sample suggests that the microbial community is more diverse.

Table 7: Distinct bacterial/archaeal OTUs in water and bioaerosol samples

| Sample Type | Distinct OTUs |
|---|---|
| Water | 264 |
| Bioaerosol – BioSampler | 132 |
| Bioaerosol – Button Aerosol Sampler | 138 |

In addition to comparisons between bioaerosol samples and water, the effect of water temperature, air exchange rate, and sampling duration on bioaerosols recovered from each bioaerosol sample type (e.g., BioSampler and Button Aerosol Sampler) were considered. Figure 8 shows PCoA plots for the microbial communities recovered from the BioSampler and Button Aerosol Sampler bioaerosol samples before and during shower operation for all six sampling events. Figure 8A shows that for the samples collected in the BioSampler, sampling event 6 yielded the most phylogenetic distinction between the microbial communities recovered in the before-shower samples versus those recovered during the shower. Sampling event 3 yielded the least phylogenetic distinction between the before- and during-shower bioaerosol samples. The bioaerosol samples collected during sampling event 6 were collected for the longest duration (45 minutes) minutes and at the lowest air exchange rate (2.5 $hr^{-1}$). This might have resulted in the collection of a during-shower bioaerosol sample with greater phylogenetic distinction from the before-shower bioaerosol sample as a longer sampling duration allows for the collection of more biomass, which might provide a more robust sample. In addition, the lower air exchange rate allowed aerosols generated during shower operation a longer residence time in the chamber as compared to higher air exchange rates which might have allowed the bioaerosol sampler to collect more of these particles. The bioaerosols collected during sampling event 3 had the shortest sampling duration (15 minutes) and the highest air exchange rate (8 $hr^{-1}$). This

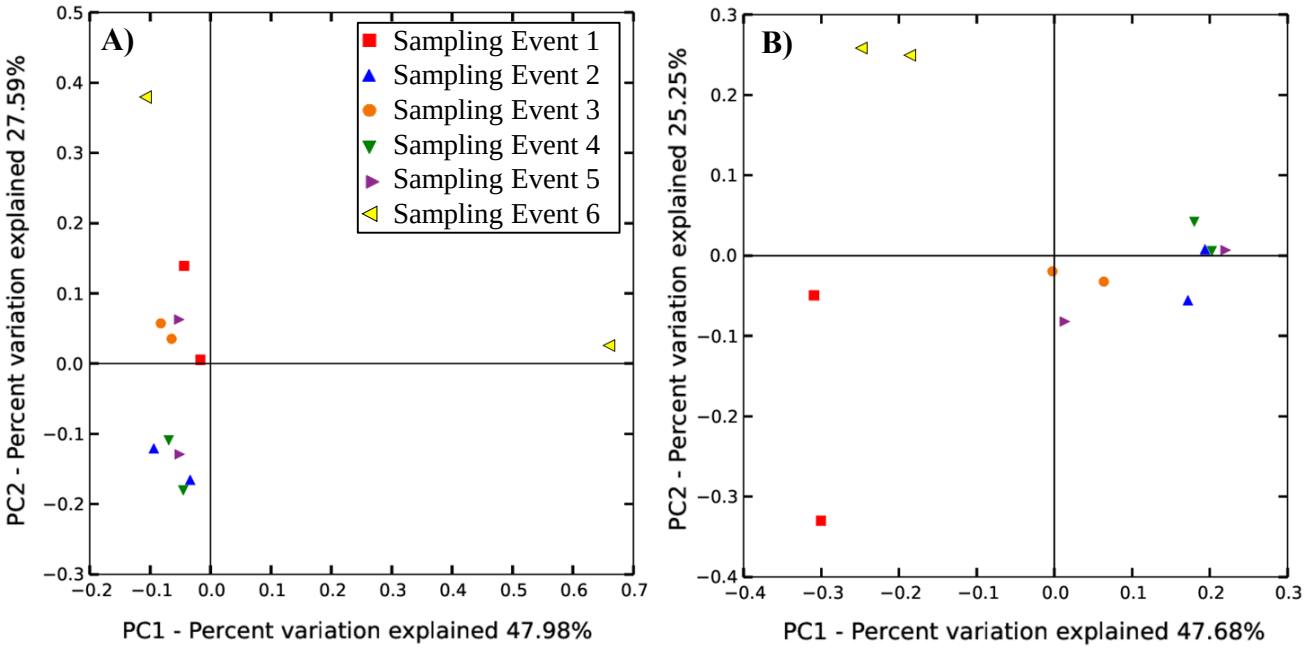


Figure 8: Weighted UniFrac PCoA plots of BioSampler bioaerosol samples (A) and Button Aerosol Sampler bioaerosol samples (B) for each sampling event

shorter sampling duration would not have allowed for the collection of as much biomass as the longer sampling duration. In addition, the higher air exchange rate might have provided a more dilute bioaerosol samples as bioaerosols generated during shower operation were removed more quickly due to the increased ventilation rate.

Figure 8B does not show the same trends observed in Figure 8A. It is possible that the low flow rate of the Button Aerosol Sampler (4 L/min compared to the BioSampler with a flow of 12.5 L/min) did not allow for the collection of bioaerosol samples with enough biomass to detect differences between the parameters varied in each sampling event. Additionally, it is possible that the Button Aerosol Sampler did not capture as many of the aerosolized water droplets (which might have contained microorganisms) as the BioSampler because the Button Aerosol Sampler collects only particles <100 µm in aerodynamic diameter. The inside diameter of the inlet to the BioSampler is 3/8 of an inch allowing this bioaerosol sampler to collect larger particles than the Button Aerosol Sampler.

Based on the trends observed in Figure 8 it could be hypothesized that a longer sampling duration with a higher throughput bioaerosol sampler could provide samples with better resolution. To this end, it is recommended to utilize a higher-throughput sampling device in further chamber recirculating shower studies, such as a WWC that samples at 100 L/min. In addition, higher air exchange rates might result in bioaerosol sampling results that more closely resemble the air being drawn into the room and not the bioaerosolization occurring during shower operation. However, considerations on the effects of flow rate, sampling duration, and chamber air exchange rate are only hypotheses based on the data collected to date. Further testing is required to test these hypotheses.

The data presented in this section indicate that the microbial communities present in the water and the bioaerosols are different. It is not clear from the data collected to date, however, whether these differences are due to the fact that the microorganisms present in the water do not aerosolize and thus are not detected in the bioaerosol samples, or whether the sampling methodology utilized (e.g., relatively low-flow samplers for short duration shower events) was unable to detect these changes. The high background microbial diversity present in the experimental chamber before shower operation also might have masked changes in the bioaerosol communities present during shower operation. Another limitation is that the recirculating experimental shower setup used in this research lacked an obstruction. Occupants in residential showers provide an obstruction that changes the spray pattern of water during a shower, which might affect aerosolization of the spray (and microorganisms within) in a manner not represented in the present study. Additionally it is important to note that the DNA extraction methods used in sampling event 6 differed from the DNA extraction method used for the other 5 sampling events. This could have also impacted the results presented in this section.

## 4.3 Chamber Physical Shower Characteristics Experiments

The effects of air exchange rate and water temperature on the particle count and particle size distribution during a shower were observed using the recirculating experimental shower in a chamber with controlled air exchange rate. Figure 9 shows the

particle size distributions in the chamber for a range of air exchange rates for both a hot (~40˚C) and cold (~24˚C) shower before the shower was turned on and while it was

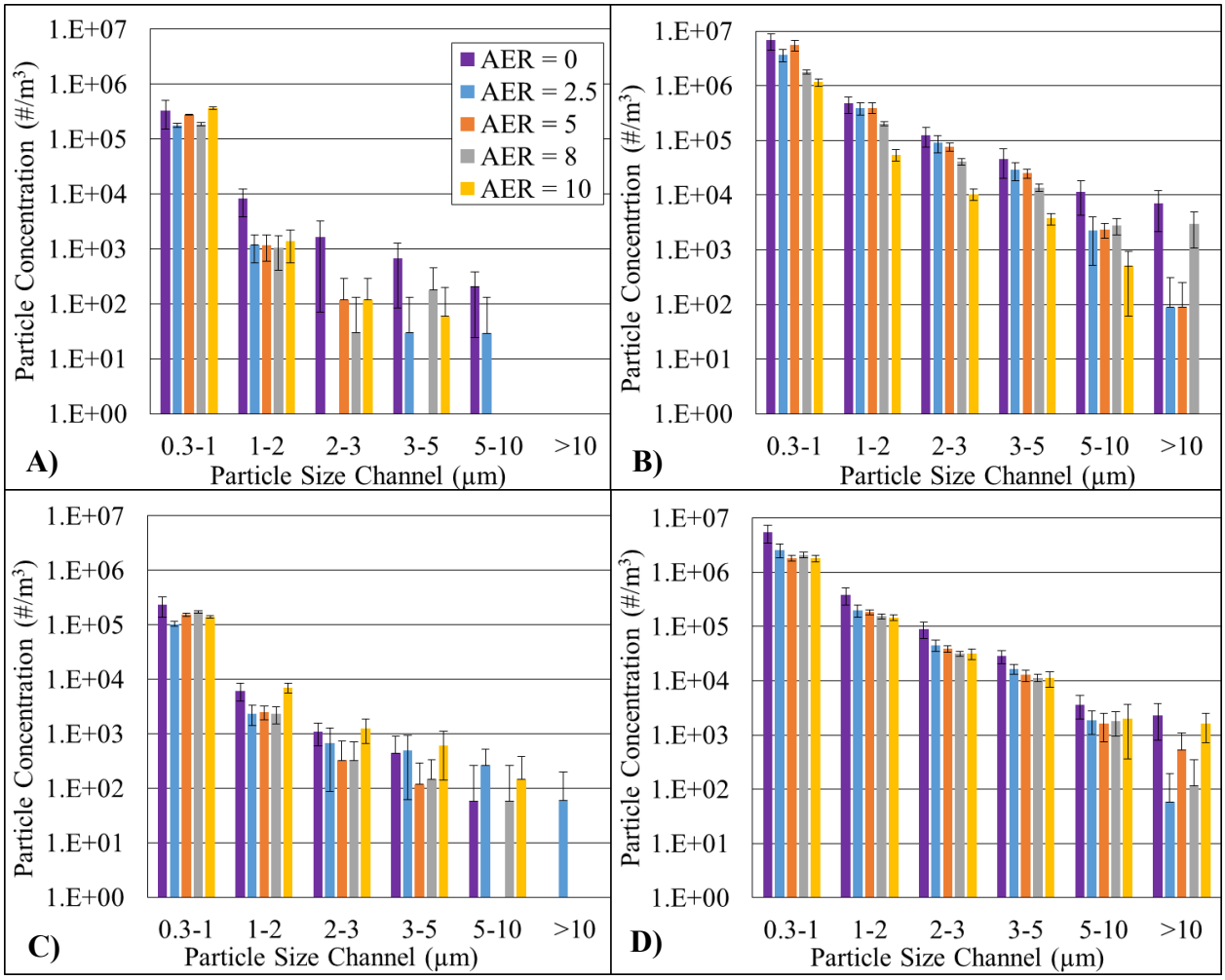


Figure 9: Particle size distribution (A) before a hot* shower, (B) during a hot shower, (C) before a cold* shower, and (D) during a cold shower. *A hot shower is defined as ~40˚C while a cold shower is defined as ~24˚C.

operating. For both the hot and cold showers, there is approximately an order of magnitude increase in particle concentration across all of the size channels and all of the air exchange rates when the shower is operating compared to when it is not operating. This indicates that when you are showering, you are exposed to about a 10-fold increase in the number of inhalable particles relative to the level when the shower is not operating.

In addition, both before and during shower operation, the majority of the particles observed in the chamber occurred in the smallest channel (0.3-1 µm). This finding is consistent with a study by Xu and Weisel (2003) who found that the majority of particles generated during a shower are 0.3 µm or smaller. These smaller particles have the potential to be problematic from a health standpoint as particles smaller than 1-5 µm in diameter can travel deeper into the human respiratory tract than larger particles (greater than 10 µm in diameter) (Thomas et al., 2008). Furthermore, it is feasible that microorganisms or microbial fragments could be present in these small particles adding to the potential health risk. It is important to note, however, that not all particles that are inhaled will deposit in the lungs; some of the particles that are inhaled will exit the human lungs during exhalation.

Additionally, during shower operation for both the hot and cold showers there is a trend of increasing particle concentrations with decreasing air exchange rates (Figures 9B and 9D). This trend is expected as a higher air exchange rate indicates that the air in the chamber is being replaced with new HEPA-filtered air more rapidly. Between the hot and cold showers, however, there are no apparent differences between the particle size distributions (Figures 9B and 9D). This indicates that increasing the temperature of shower water from 24˚C to 40˚C does not have a significant effect on the concentration of inhalable particles generated during shower operation.

It is important to mention the limitations of the particle size distribution data reported in this study (Figure 9). The TSI Aerotrak is a device that is meant to measure dust particles, and the device is calibrated to particles that mimic airborne dust. Additionally, the size of water particles by nature are difficult to accurately measure as they evaporate in air resulting in a dynamic diameter size. For these reasons, there could be errors associated with the particle size distribution data presented in this study, and the data should only be used for comparative purposes among experimental conditions and as a rough estimate of the sizes of particles present.

Figure 10A shows that by increasing the water temperature by 10˚C (and holding the air exchange rate constant at 2.5 $h^{-1}$) in the experimental shower, the peak relative humidity during shower operation increased by over 10%, and the temperature increased

by almost 5˚C. In addition, the air exchange rate was also found to affect the relative humidity in the room. Increasing the air exchange rate from 2.5 $h^{-1}$ to 8 $h^{-1}$ (holding the water temperature constant at 40 ˚C) resulted in a decrease in the peak relative humidity by almost 10% with the air temperature remaining relatively constant.

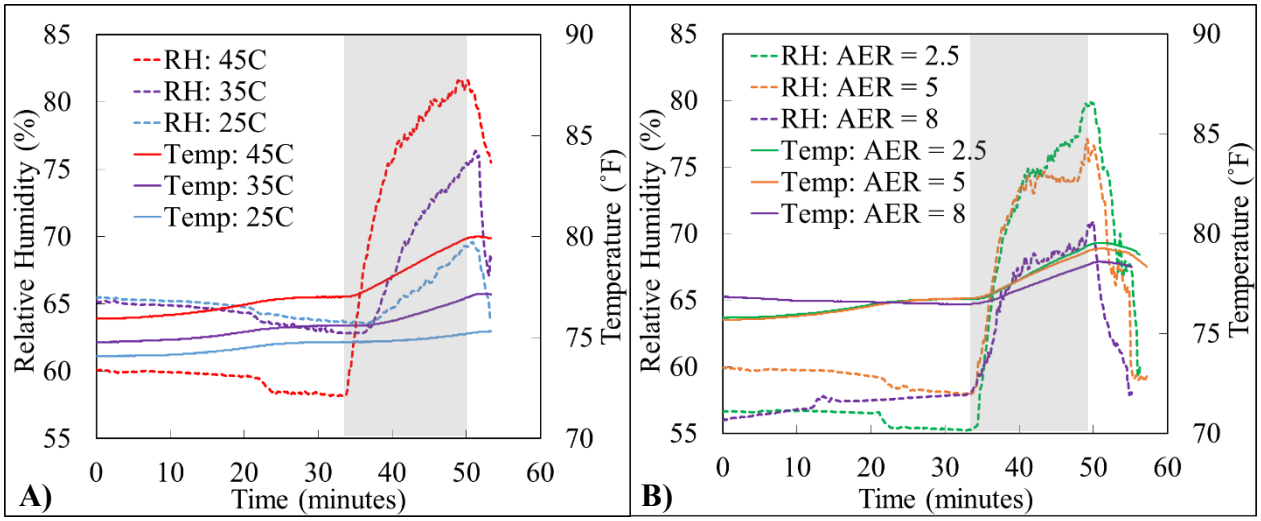


Figure 10: Relative humidity and temperature for (A) increasing shower water temperatures and (B) increasing air exchange rates. Dotted lines indicate relative humidity and solid lines indicate temperature. The grey rectangle covering a portion of each graph indicates the times during which the shower was operating.

These results suggest that the physical conditions within residential showers are variable. A few degrees change in the temperature of the water during a shower or the use of a fan (which increases the bathroom's air exchange rate) can change the temperature, relative humidity, and number of inhalable particles to which you are exposed. These findings suggest that shower occupants should utilize their bathroom fans while showering to reduce the number of inhalable particles to which they are exposed.

# CHAPTER 5. SUGGESTIONS FOR FUTURE RESEARCH

Suggestions for future research relating to residential shower sampling and data analysis are reported in this section. First, a suggestion for additional *Mycobacterium* species to target in residential shower samples via qPCR is provided. Second, further Chamber Tap Water Bioareosol Experiments are recommended to delineate the effects of tap water on shower bioaerosols. Third, an experiment demonstrating the use of relative humidity data as a proxy for shower duration and usage is presented and suggestions for incorporating this technique into further research are provided.

## 5.1 Detection and Quantification of *M. gordonae* and *M. canariasense*

The presence of *M. avium* complex in residential shower samples collected from both the Austin residence and the San Antonio residence was investigated using qPCR. However, the presence of this bacterial complex was not identified in any residential shower sample to the detection limit of 29 gene copies/µL. To determine what species from the *Mycobacterium* genus might be present in these samples, reference sequences chosen by QIIME to identify the *Mycobacterium* genus in our residential shower samples were compared to species-specific databases used in BLASTn to look for isolates with 16S rRNA sequences that were >97% similar. The two species from this analysis that were determined most suitable for further investigation using qPCR were *M. gordonae* and *M. canariasense* (see section 4.1). These species were chosen due to their high similarity to the reference sequences, the fact that their isolate source environments were tap-water-related, and the fact that both species are of public health concern. It is recommended for future research to design qPCR assays to examine the presence and quantity of these two mycobacterial species in residential shower samples from Austin and San Antonio.

## 5.2 Future Chamber Tap Water Bioaerosol Experiments

The Chamber Tap Water Bioaerosol Experiments presented in Section 4.2 were intended to investigate the contribution of microorganisms in tap water to the microbial communities present in shower aerosols. However, limitations to this investigation

prevented clear conclusions from being drawn. Future Chamber Tap Water Bioaerosol Experiments are recommended to complement the results presented in Section 4.2. First it is recommended that before further sampling events are conducted that the HEPA air filter at the air intake to the chamber be replaced. It is then recommended to run triplicate sampling events utilizing a high-throughput WWC (100 L/min) bioaerosol sampling device for both 15- and 45-minute sampling durations at an air exchange rate of 2.5 $hr^{-1}$. It is also advised to include an obstruction (for example, a mannequin) in the recirculating experimental shower to obtain a more realistic spray pattern during shower operation. Running these additional sampling events should provide insight into the impacts of sampler flow-rate, sampling duration, and the inclusion of an obstruction on the ability to detect differences in bioaerosol communities present before and during shower operations.

## 5.3 Tracking Shower Usage Based on Relative Humidity for Future Residential Sampling Events

Single shower events in a home in Austin, TX, were investigated to determine whether relative humidity could serve as a proxy for shower duration and usage. This topic was studied as shower duration and usage might impact biofilm formation in shower plumbing and on shower surfaces which might, in turn, affect the microbial communities aerosolized during shower operation. Presented in this section are an experimental summary investigating this topic and applications of this investigation for future residential sampling events.

### METHODS

Three relative humidity and temperature logging devices (HOBOs) were positioned in an Austin, TX, bathroom in three different locations to try and capture relative humidity variation across the bathroom space during a showering event. One HOBO was located inside of the shower on the opposite wall from the showerhead approximately 4 inches below the ceiling ("Shower HOBO" in Figures 11-13). The second HOBO was located on the bathroom sink counter on the middle of the edge closest to the shower approximately 2 feet above the ground ("Sink HOBO" on the Figures 11-13). The third was located above

the door on the inside of the bathroom approximately three inches below the ceiling ("Door HOBO" in Figures 11-13). Relative humidity was measured continuously at 5-second intervals for the duration of each experiment. Three different showering conditions were investigated for their effect on relative humidity readings: (1) fan on, occupied; (2) fan off, occupied, and (3) fan on, unoccupied. 'Occupied' indicates a showering event where a human was present in the shower during data collection while 'unoccupied' indicates a showering event where there was no human obstruction present in the shower during data collection. Figures 11-13 show relative humidity over time at three different locations in a bathroom during the three different experiments.

## RESULTS AND DISCUSSION

### Optimal location of HOBOs

The sink HOBO consistently recorded lower relative humidity than the other two HOBOs, most likely due to the location of the HOBO. The door and shower HOBOs were located very close to the ceiling while the sink HOBO was located only a couple of feet above the ground. Since hot air rises, the HOBOs located closer to the ceiling recorded higher relative humidity than did the sink HOBO located closer to the floor.

In addition, the sink HOBO was the only HOBO of the three that showed an increase in relative humidity after the shower was turned off for the two occupied experiments (Figures 11 and 12). The unoccupied shower experiment (Figure 13), however, showed a decrease in relative humidity after the shower was turned off for all three HOBOs. This increase in relative humidity for the sink HOBO after the shower was turned off during the experiments shown in Figures 11 and 12 was likely due to the shower occupant exiting the shower. As the occupant exited the shower, they opened the shower curtain, removing a partition between the more humid shower air and the less humid air in the bathroom. In addition, the occupant themselves upon exiting the shower moved closer to the sink to dry off, bringing the humidity around their body closer to the sink HOBO. The other two HOBOs were far enough away and already registering a much higher relative

humidity than the sink HOBO at the same time and therefore were not affected by the occupant exiting the shower.

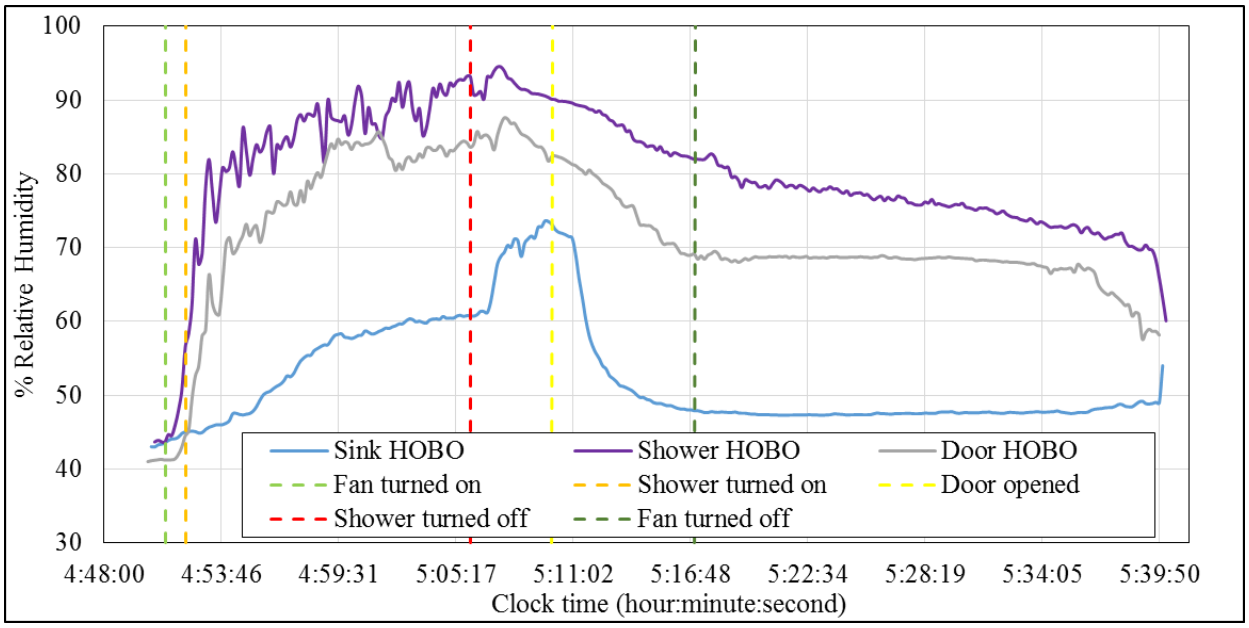


Figure 11: Relative humidity during an occupied shower with the fan on.

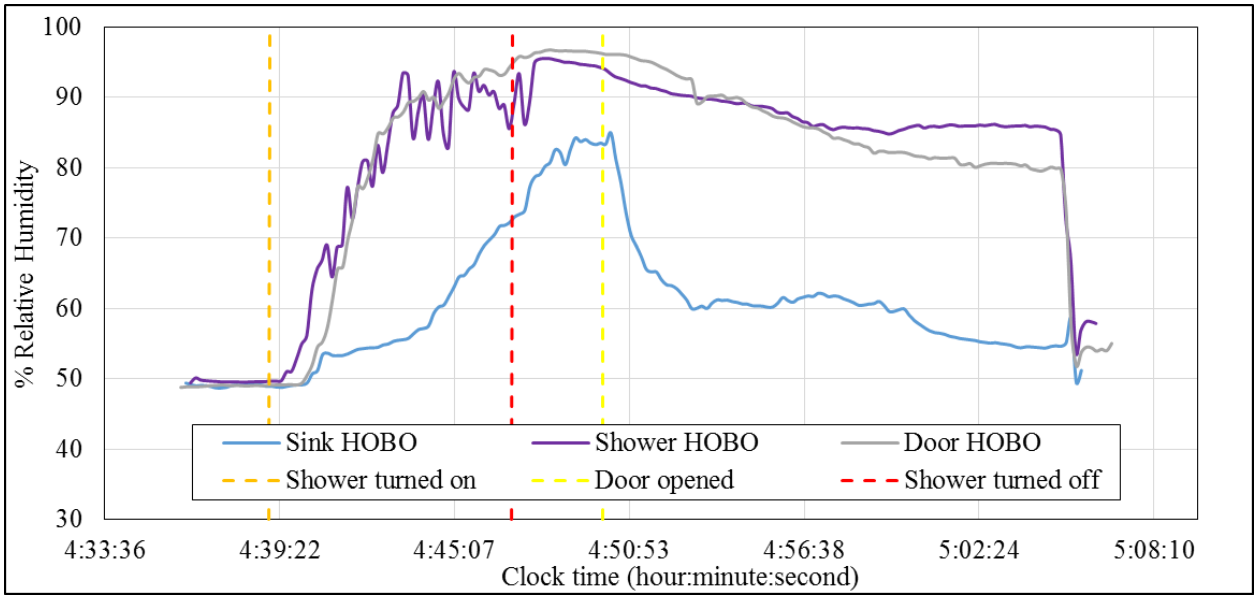


Figure 12: Relative humidity during an occupied shower with the fan off.

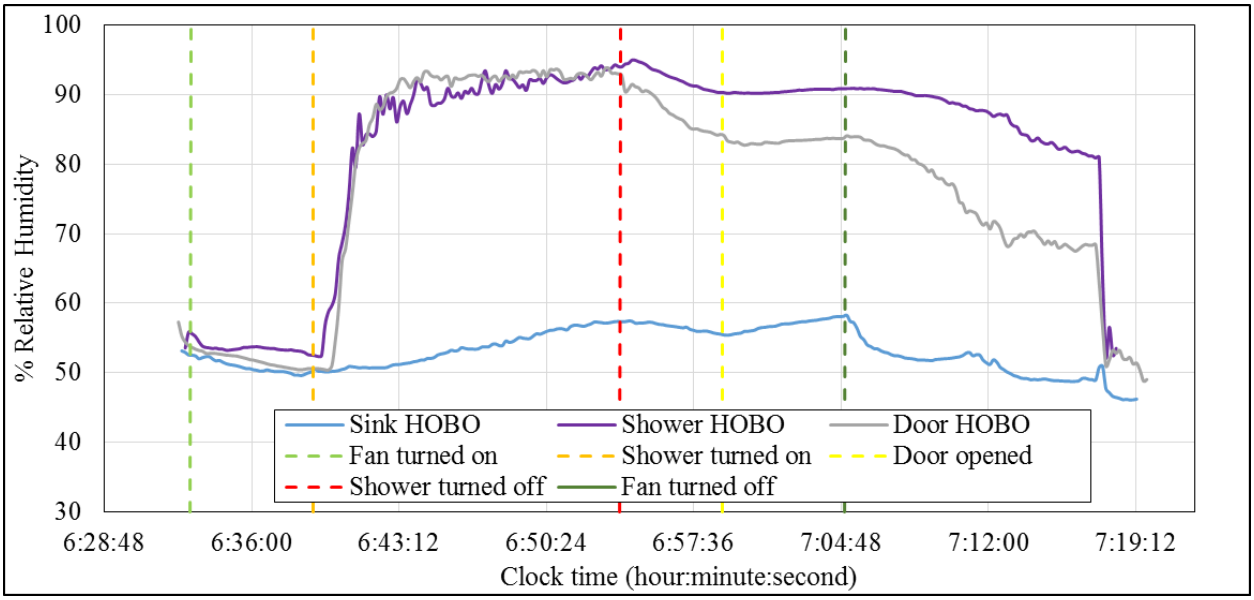


Figure 13: Relative humidity during an unoccupied shower with the fan on.

As the sink HOBO showed less consistent relative humidity readings than the shower or door HOBOS, it appears that the shower or door location is more appropriate to monitor relative humidity as an indicator of shower water usage. The shower and door HOBOs recorded a trend in relative humidity that was more expected with relative humidity increasing significantly during the shower, and decreasing when the shower was no longer operating.

**Change in relative humidity as an indicator of shower duration and water usage**

For all three experiments the shower and door HOBOs showed a significant increase in relative humidity immediately after turning on the shower. Because the relative humidity increased at such a fast rate once the shower was turned on and was relatively constant before then, the start of the shower was estimated as the time at which the relative humidity first reaches a value closest to 110% of the initial relative humidity. This calculation is shown in Equation 2.

$$Shower\ Start\ Time = t(RH_{initial} * 1.10) \quad \text{(Eq. 2)}$$

Where $RH_{initial}$ is the first relative humidity data point recorded by the HOBO. The factor of 1.10 was chosen as a 10% increase above the initial relative humidity was never achieved before the start of the shower, and as this value allows us to avoid interpreting any fluctuations in relative humidity before the shower starts as a false start time.

The maximum relative humidity for the door and shower HOBOs in each of the three experiments occurred just after the shower was turned off, and, as such, was used to estimate of the end of the shower as shown in Equation 3:

$$Shower\ Stop\ Time = t(RH_{max}) \quad \text{(Eq. 3)}$$

Using these estimates of the start and stop times of a shower, the duration of a shower was estimated from relative humidity data. These data are shown in Table 8, and estimated shower duration are compared to the actual shower durations for each experiment.

Table 8: Shower duration estimations based on relative humidity data from a HOBO located near the door of a bathroom and in the shower. Data is reported in the format minutes:seconds.

| Experiment | Actual Shower Duration | Door HOBO | | Shower HOBO | |
|---|---|---|---|---|---|
| | | Estimated Shower Duration | Difference | Estimated Shower Duration | Difference |
| 1 | 14:00 | 15:30 | 1:30 | 15:50 | 1:50 |
| 2 | 8:00 | 7:40 | 0:20 | 8:10 | 0:10 |
| 3 | 15:00 | 13:00 | 2:00 | 14:50 | 0:10 |

Based on the data shown in Table 8 it appears that both the wall opposite of the showerhead and above the door of the bathroom are both acceptable locations for a HOBO to record relative humidity data. Estimating shower duration with relative humidity data using this method appears to be a useful proxy. As shown in Table 8, in every experiment this estimation was never off by more than two minutes.

In addition to estimating shower duration, these data could easily be used to estimate water usage during a shower, provided that the flow rate was known. This flow rate could be calculated by timing how long it takes for water exiting the showerhead to

fill a container of known volume and then dividing the volume of the container used by the time it took to fill that container. Multiplying the flow rate by the duration of the shower would then provide an estimate for water usage during a shower.

### APPLICATIONS FOR FUTURE RESEARCH

The results of this investigation will be useful for future residential shower sampling events. By employing a HOBO data logger in residential showers a week or more before a sampling event, the researcher could record useful information regarding shower usage. This information could then be used to look for correlations between shower usage and aerosolized microbial communities or biofilm communities on surfaces.

However, for relative humidity data collected over several days, the calculations presented in this section could only be used if the researcher first isolated individual shower events. Alternatively, a computer programming language, such as R which is free and available online, could be used to filter through relative humidity data over several days using a predetermined ‘shower on’ and ‘shower off’ condition to calculate the number and duration of showers.

# CHAPTER 6. CONCLUSIONS

This research investigated characteristics of aerosols generated during shower operation in two different environments: residential showers and a controlled experimental shower set-up. These two environments allowed for both the characterization of microbial aerosols in regularly used showers with existing biofilms, and the characterization of microbial aerosols in a simulated shower with no biofilm growth, respectively. In addition, the controlled experimental shower set-up was used to determine the effects of air exchange rate and shower water temperature on physical shower characteristics. The specific aims of this study were to detect the presence and quantity of selected species in residential showers that could potentially pose a health risk to occupants. Additionally, this research sought to elucidate the impact of tap water microorganisms on shower aerosols, and the impact of air exchange rate and water temperature on shower aerosols, ambient air temperature, and relative humidity in bathrooms. Specific conclusions from this research are as follows:

1. An allergenic fungal species, *A. alternata*, was detected in both of the residential showers investigated in this study. *A. alternata* was detected in six aerosol samples from a residence in Austin, TX (three samples from inside of the shower before, during and after operation and three sequential samples from a room adjacent to the shower) and three aerosol sample from a residence in San Antonio, TX (inside of the shower during and after operation, and one sample from a room adjacent to the shower). Additionally, *A. alternata* exhibited the highest gene copy concentrations during shower operation at both residences. However, based on the data presented there is not enough evidence to draw conclusions regarding the source of this microorganism in the two residential showers.
2. A complex of opportunistic human pathogens, *M. avium* complex, was not detected in either residential shower investigated in this study to the detection limit of 29 gene copies/µL. Based on 16S rRNA gene amplicon sequence similarity of *Mycobacterium* species to reference sequences from the residential shower

samples, it might be appropriate to target *M. gordonae* and *M. canariasense* for further research.

3. Experiments run in a recirculating experimental shower indicated that tap water contained substantially different and more diverse microbial communities than did bioaerosols collected both before and during shower operation. Using the experimental shower set up and low-flow bioaerosol samplers employed in this study, the bioaerosol communities were dissimilar to the water microbial communities.
4. The choice of biosampler type and sampling duration might substantially impact the bioaerosol communities collected. It is possible that a longer sampling duration and higher-throughput sampling device could provide more robust bioaerosol samples. Additionally, better filtration of outside air drawn into the chamber would allow for lower background levels of bioaerosols, which might allow for better detection of the bioaerosolization of tap water during shower operation.
5. The shower environment exposes occupants to ten-fold increase in the concentration of inhalable particles across a variety of sizes (0.3 μm to >10 μm in diameter) during shower operation as compared to before shower operation.
6. The concentration of particles to which shower occupants are exposure during shower operation and the relative humidity in a bathroom both decrease with increasing bathroom air exchange rate.
7. An increase in water temperature during a shower from 25-40˚C does not have a substantial effect on the concentration of particles we are exposed to during a shower. An increase in water temperature does, however, substantially increase the relative humidity and air temperature in a bathroom.

The results presented in this thesis add to the current literature regarding the characterization of the shower microbiome from both a physical and biological perspective. Additionally, suggestions for future research (Chapter 5) provide the basis for further investigations on the topics presented herein.

# CHAPTER 7. APPENDICES

## 7.1 Quant-iT dsDNA, High Sensitivity Standard Curve for DNA Quantification

A standard curve for the Quant-iT dsDNA, High Sensitivity DNA quantification kit was made using the *Escherichia coli* DNA standards provided in the kit. The lowest concentration DNA standard in the kit was 0.5 ng/µL, so dilutions of this standard were made using DNA-free water to achieve the lower DNA masses shown on Figure A1. There are more points on the standard curve (Figure A1) in the 1-5 ng range as the majority of samples tested had a DNA mass of less than 2 ng in the 2 µL that were used for this analysis.

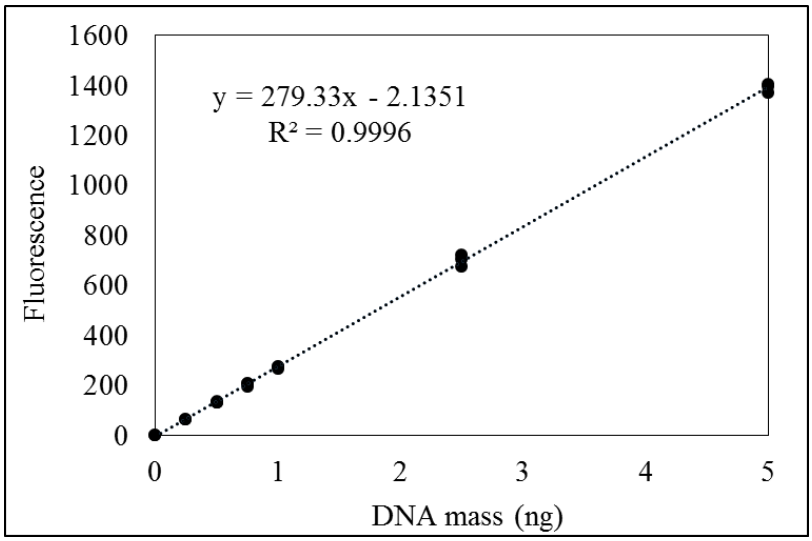


Figure A1: Quant-iT dsDNA standard curve for DNA masses from 0-5 ng

## 7.3 qPCR for *A. alternata*

An example of a qPCR standard curve for *A. alternata* is shown in Figure A2.

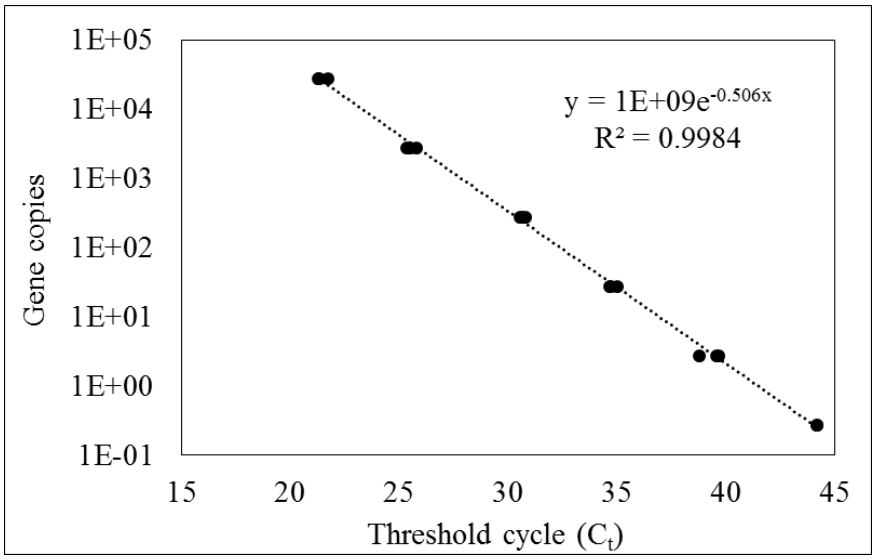


Figure A2: qPCR standard curve for *A. alternata*

The number of gene copies per reaction for each standard was calculated using Equation A1:

$$\frac{gene\ copies}{\mu L} = \left(DNA\ concentration\ of\ standard\ \left[\frac{ng}{\mu L}\right]\right) \times \left(\frac{6.023\times10^{23} bp\ DNA}{1\ mole\ DNA}\right) \times \left(\frac{1\ gene\ copy}{33{,}200{,}000\ bp\ in\ the\ A.alternata\ genome*}\right) \times \left(\frac{1\ mole\ DNA}{660\ g}\right) \times \left(\frac{1\ g}{10^9\ ng}\right) \times (1\ \mu L\ standard\ DNA\ added\ to\ reaction) \quad \text{(Eq. A1)}$$

*The number of gene copies in the *A. alternata* genome and the size of the genome were determined using the fully sequenced *A. alternata* (ATCC 66981) genome by Dr. Christopher Lawrence, Department of Biological Sciences, Virginia Bioinformatics Institute, Blacksburg, VA (the publication containing this information is currently under review).

DNA concentrations ranging from 0.3 to $3E\times10^4$ gene copies/µL were used to create the *A. alternata* standard curves.

**LIMIT OF DETECTION**

The limit of detection for this qPCR method was determined as the lowest concentration at which 95% of samples positively amplified (Brookman-Amissah et al., 2012; Bustin et al., 2009). Out of fifteen replicates of *A. alternata* genomic DNA (the positive control DNA used for this method) the lowest standard, 0.3 gene copies/µL, amplified 67% of the time while the next lowest standard, 3 gene copies/µL, amplified 100% of the time. To determine the concentration where *A. alternata* genomic DNA amplified 95% of the time, 15 replicates of this genomic DNA were run at standard concentrations between the two lowest standards: 0.975, 1.65, and 2.325 gene copies/µL. The 2.325 gene copies/µL standard amplified 93% of the time. As this was close to 95% and the next highest standard concentration, 3 gene copies/µL, amplified 100% of the time, a linear interpolation between these two concentrations was used to determine the concentration at which *A. alternata* genomic DNA amplified 95% of the time, or the Limit of Detection. This calculation is shown in Equation A2:

$$Limit\ of\ Detection = 3\,\frac{gene\ copies}{\mu L} - \frac{(100\%-95\%)\left(3\frac{gene\ copies}{\mu L} - 2.325\frac{gene\ copies}{\mu L}\right)}{(100\%-93\%)} \quad \text{(Eq. A2)}$$

Based on this calculation, the concentration at which *A. alternata* amplifies 95% of the time is 2.5 gene copies/µL making this the limit of detection for this qPCR method.

## 7.4 qPCR for *M. avium* complex

**STANDARD CURVE**

An example of a qPCR standard curve for *M. avium* complex is shown in Figure A3. DNA from a species of the *M. avium* complex, *M. avium* subsp. *paratuberculosis*, was used to generate the standard curve.

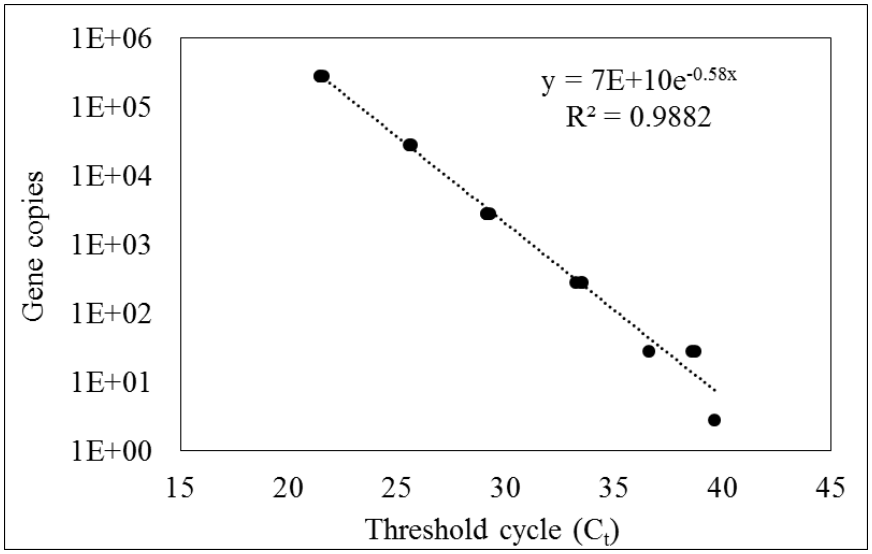


Figure A3: *M. avium* complex specific qPCR standard curve

The number of gene copies per reaction for each standard were calculated using Equation A3:

$$\frac{gene\ copies}{\mu L} = \left(DNA\ concentration\ of\ standard\ \left[\frac{ng}{\mu L}\right]\right) \times \left(\frac{6.023\times10^{23}\ bp\ DNA}{1\ mole\ DNA}\right) \times \left(\frac{1\ gene\ copy}{4{,}829{,}781\ bp\ in\ the\ M.\ avium\ \text{subsp.}\ paratuberculosis\ \text{genome}*}\right) \times \left(\frac{1\ mole\ DNA}{660\ g}\right) \times \left(\frac{1\ g}{10^9\ ng}\right) \times (1.5\ \mu L\ standard\ DNA\ added\ to\ reaction) \qquad \text{(Eq. A3)}$$

*The number of gene copies per *M. avium* subsp. *paratuberculosis* was determined by searching the full genome FASTA file for the region being amplified using primer sequence reported in the Methods section of this study. The genome size was found as reported by the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/nuccore/AE016958.

DNA concentrations ranging from 3 to 3E×$10^5$ gene copies/µL were used to create the *M. avium* complex standard curves.

**LIMIT OF DETECTION**

The limit of detection for this qPCR method was determined as the lowest concentration at which 95% of samples positively amplified (Brookman-Amissah et al., 2012; Bustin et al., 2009). Out of twelve replicates of *M. avium* subsp. *paratuberculosis* genomic DNA (the positive control DNA used for this method) the lowest standard, 3 gene copies/µL, amplified 58% of the time while the next lowest standard, 30 gene copies/µL, amplified 100% of the time. To determine the concentration where *M. avium* subsp. *paratuberculosis* genomic DNA amplified 95% of the time, 12 replicates of this genomic DNA were run at standard concentrations between the two lowest standards: 9.75, 16.5, and 23.25 gene copies/µL. The 23.25 gene copies/µL standard amplified 67% of the time. As this was the closest to 95% other than the next highest standard concentration, 3 gene copies/µL which amplified 100% of the time, a linear interpolation between these two concentrations was used to determine the concentration at which *M. avium* subsp. *paratuberculosis* genomic DNA amplified 95% of the time, or the Limit of Detection. This calculation is shown in Equation A4:

$$Limit\ of\ Detection = 30\,\frac{gene\ copies}{\mu L} - \frac{(100\%-95\%)\left(30\frac{gene\ copies}{\mu L}-23.25\frac{gene\ copies}{\mu L}\right)}{(100\%-67\%)} \quad \text{(Eq. A4)}$$

Based on this calculation, the concentration at which *A. alternata* amplifies 95% of the time is 29 gene copies/µL making this the limit of detection for this qPCR method.

## 7.5 Rarefaction Curves for Residential Shower Samples and Chamber Tap Water Bioaerosol Experiment Samples

Figure A5 shows the rarefaction curves for bacterial/archaeal sequences from samples collected from the residential shower in Austin, TX. The different legend items map to a Sample Name as shown in Table 4 with the following two exceptions: (1) The legend item 'Plumbing Biofilms' includes the samples 'Sonicated showerhead liquid, External showerhead swab, and Internal pipe swab' from Table 4, and (2) the legend item Surface biofilms includes the samples 'Wall swab, External showerhead swab, Internal pipe swab, Ceiling swab, and Floor swab' from Table 4. The water sample rarefaction curve begins leveling off later than all of the other sample rarefaction curves. This indicates

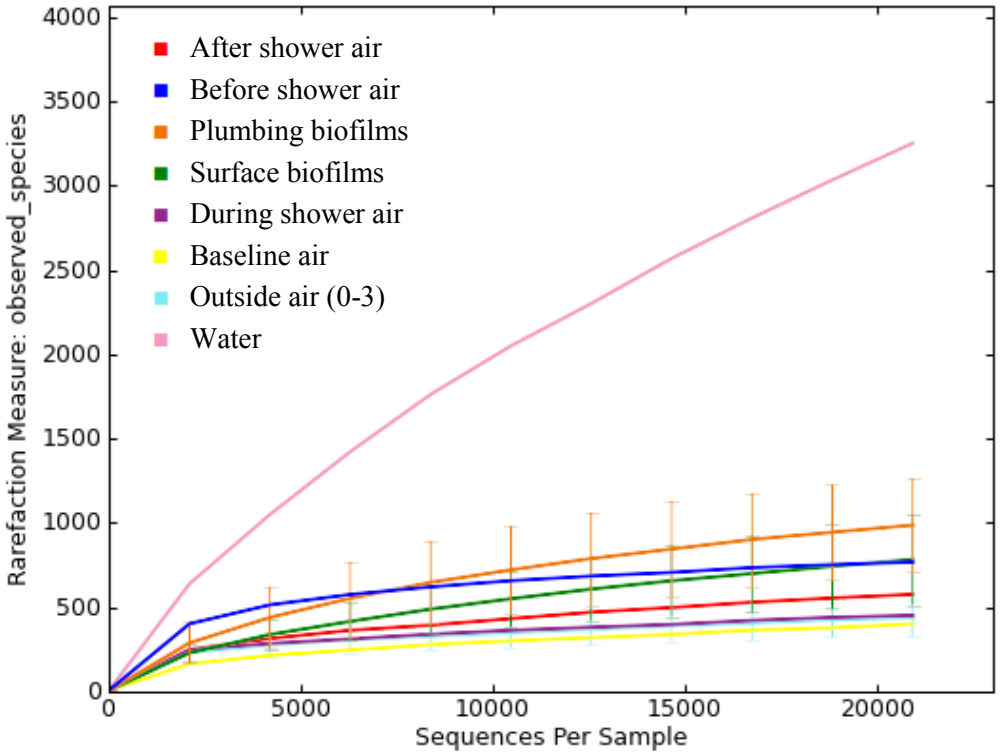


Figure A4: Rarefaction curves for *Bacteria/Archaea* sequencing results from the Austin, TX, residential shower samples

that the water sample contains greater diversity than any other sample. Error bars indicate variation between groups of samples included in each legend item. All of the samples sequenced for *Bacteria/Archaea* from the Austin residential shower were rarefied to 20,913 sequences per sample.

Figure A6 shows the rarefaction curves for fungal sequences from samples collected from the residential shower in Austin, TX, and the residential shower in San Antonio, TX. The different legend items map to a Sample Name as shown in Table 4 with the same exceptions mentioned for Figure A5. Error bars indicate variation between groups of samples included in each legend item. All of the samples from the Austin residential

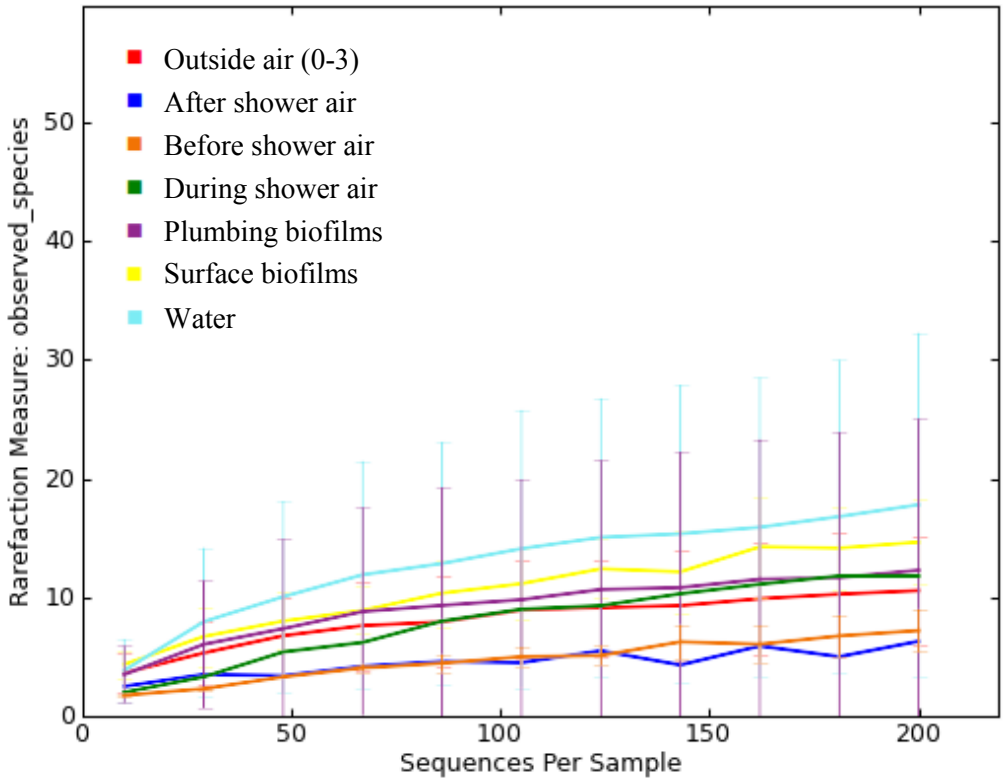


Figure A5: Rarefaction curves for *Fungi* sequencing results from the Austin, TX, and San Antonio, TX, residential shower samples

shower and the San Antonio residential shower that were sequenced for *Fungi* were rarefied to 200 sequences per sample.

Figure A7 shows the rarefaction curves for bacterial/archaeal sequences from samples collected during the Chamber Tap Water Bioaerosol Experiments. From these experiments, the water rarefaction curve also begins to taper off more slowly than the other samples indicating greater diversity. The BioSampler and Button Aerosol Sampler lines on this figure represent all of the bioaerosol samples collected using these two bioaerosol sampling devices with error bars representing among samples. Similarly, variation between water samples are shown in the error bars for that sample type. All of the samples from the Chamber Tap Water Bioaerosol Experiments were rarefied to 12,152 sequences per sample.

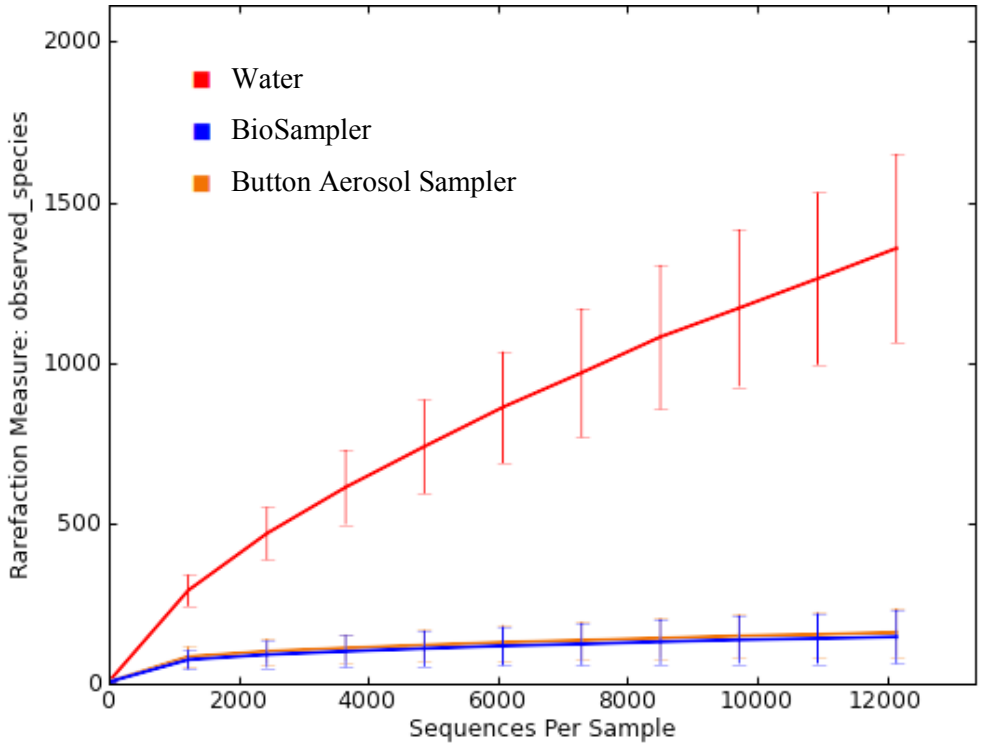


Figure A6: Rarefaction curves for *Bacteria/Archaea* sequencing results from the Chamber Tap Water Bioaerosol Experiment Samples

# CHAPTER 8. REFERENCES


Altschul, S., Gish, W., Miller, W., Myers, E., & Lipman, D. (1990) "Basic Local Alignment Search Tool," Journal of Molecular Biology, 215(3), 403-410.

Anaissie, E. J., Stratton, S. L., Dignani, M. C., Summerbell, R. C., Rex, J. H., Monson, T. P., Spencer, T., Kasai, M., Francesconi, A., & Walsh, T. J. (2002) Pathogenic Aspergillus species recovered from a hospital water system: a 3-year prospective study. *Clinical Infectious Diseases, 34*(6), 780–789.

Angenent, L. T., Kelley, S. T., St Amand, A., Pace, N. R., & Hernandez, M. T. (2005) Molecular identification of potential pathogens in water and air of a hospital therapy pool. *Proceedings of the National Academy of Sciences of the United States of America, 102*(13), 4860–4865.

Babiceanu, M. C., Howard, B. a, Rumore, a C., Kita, H., & Lawrence, C. B. (2013) Analysis of global gene expression changes in human bronchial epithelial cells exposed to spores of the allergenic fungus, *Alternaria alternata*. *Frontiers in Microbiology*, 4, 1-14.

Baker, G. C., Smith, J. J., & Cowan, D. A. (2003) Review and re-analysis of domain specific 16S primers. *Journal of Microbiological Methods*, 55, 541–555.

Bays, L. R., Burman, N. P., & Lewis, W. M. (1970) Taste and odour in water supplies in Great Britain: a survey of the present position and problems for the future. *Water Treatment and Examination*, 19, 136–160.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauserud, H. (2010) ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BCM Microbiology, 10(189), 1-9.

Berry, D., Xi, C., & Raskin, L. (2006) Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*, 17, 297–302.

Bokulich, N. & Mills, D. A. (2013) Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities. Applied and Environmental Microbiology, 79(8), 2519-2526.

Brookman-Amissah N., Packer, H., Prediger, E., Sabel, J., Gunstream, S., Hellemans, J., Menezes, A., Owens, B., Rose, S., Sander, R., & Vandesompele, J. (2011-2012) qPCR Application Guide: Experimental Overview, Protocol, Troubleshooting. Third Edition. Integrated DNA Technologies Inc.

Bush, R. K. & Prochnau, J. J. (2004) Alternaria-induced asthma. *Journal of Allergy and Clinical Immunology*, 113, 227-34.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, 55(4), 611–622.

Campos-Herrero, M. I., Garcia, D., Figuerola, A., Suarez, P., Campo, C., & Garcia, M. J. (2006) Bacteremia caused by the novel species *Mycobacterium canariasense*. *European Journal of Clinical Microbiology & Infectious Diseases*, 25, 58-60.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Gonzalez, P. A., Goodrich, J. K., Gordon, J. I., Huttley, G.A., Kelley, S. T., Knights, D., Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, & Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335-336.

Chang, C. W., & Hung, P. Y. (2012) Evaluation of sampling techniques for detection and quantification of airborne legionellae at biological aeration basins and shower rooms. *Journal of Aerosol Science*, 48, 63–74.

Chang, C. T., Wang, L. Y., Liao, C. Y., & Huang, S. P. (2002) Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. *Applied and Environmental Microbiology*, 68, 3159–3161.

Chen, M. J., Wu, K. Y., & Chang, L. (2003) A new approach to estimating the volatilization rates of shower water-contained volatile organic compounds during showering. *Atmospheric Environment*, 37(31), 4325–4333.

Cho, S. H., Seo, S. C., Schmechel, D., Grinshpun, S. A., & Reponen, T. (2005) Aerodynamic characteristics and respiratory deposition of fungal fragments. *Atmospheric Environment*, *39*(30), 5454–5465.

Chowdhury, S. (2012) Heterotrophic bacteria in drinking water distribution system: a review. *Environmental Monitoring and Assessment*, 184, 6087–6137.

Claesson, M. J., Wang, Q., O'Sullivan, O, Greene-Diniz, R., Cole, J. R., Ross, R. P., & O'Toole, P. W. (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research*, 38(22), 1-13.

Covert, T. C., Rodgers, M. R., Reyes, A. L., & Stelma, G. N. (1999) Occurrence of nontuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology*, 65, 2492–2496.

Cowen, K. a., & Ollison, W. M. (2006) Continuous Monitoring of Particle Emissions during Showering. *Journal of the Air & Waste Management Association*, 56, 1662–1668.

De los Reyes, F. L., & Raskin, L. (2002) Role of filamentous microorganisms in activated sludge foaming: relationship of mycolata levels to foaming initiation and stability. *Water Research*, *36*(2), 445–459.

Doggett, M. S. (2000) Characterization of Fungal Biofilms within a Municipal Water Distribution System. *Applied and Environmental Microbiology*, *66*(3), 1249–1251.

Doyle, J. J. & Doyle, J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemnical Bulletin*, 19, 11-15.

Falkinham, J. O., Norton, C. D., & LeChevallier, M. W. (2001) Factors Influencing Numbers of Mycobacterium avium, Mycobacterium intracellulare, and Other Mycobacteria in Drinking Water Distribution Systems. *Applied and Environmental Microbiology*, *67*(3), 1225–1231.

Falkinham, J. O. (2003) Mycobacterial aerosols and respiratory disease. *Emerging Infectious Diseases*, *9*(7), 763–7.

Falkinham, J. O., Iseman, M. D., Haas, P. de, & Soolingen, D. van. (2008) Mycobacterium avium in a shower linked to pulmonary disease. *Journal of Water and Health*, *6*(2), 209–213.

Feazel, L. M., Baumgartner, L. K., Peterson, K. L., Frank, D. N., Harris, J. K., & Pace, N. R. (2009) Opportunistic pathogens enriched in showerhead biofilms. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(38), 16393–16399.

Gardes, M. & Bruns, T. (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2, 113–118.

George, K. L., Parker, B. C., Gruft, H., & Falkinham III, J.O. (1980) Epidemiology of infection by nontuberculous mycobacteria. II. Growth and survival in natural waters. *American Review of Respiratory Disease*, 122, 89–94.

Girard,M.,Lacasse, Y., & Cormier, Y. (2009) Hypersensitivity pneumonitis. *Allergy*, 64, 322-334.

Globa, A. G., Alekseev, Y. I., Arzumanyan, V. G., Zaborova, V. A., & Guridov, A. A. (2013) The use of real-time PCR for quantitative determination of some propionic bacteria inhabiting the human skin. *Biomedical Chemistry*, 7(2), 175-177.

Górny, R. L., Dutkiewicz, J., & Krysinska-Traczyk, E. (1999) Size distribution of bacterial and funal bioaerosols in indoor air. *Annals of Agricultural and Environmental Medicine*, 6, 105–113.

Gomez-Alvarez, V., Revetta, R. P., & Santo Domingo, J. W. (2012) Metagenomic analyses of drinking water receiving different disinfection treatments. *Applied and Environmental Microbiology*, *78*(17), 6095–102.

Hageskal, G., Lima, N., & Skaar, I. (2009) The study of fungi in drinking water. *Mycological Research*, *113*, 165–172.

Halonen, M., Stern, D. A., Wright, A. L., Taussig, L. M., & Martinez, F. D. (1997) Alternaria as a major allergen for asthma in children raised in a desert environment. *American Journal of Respiratory and Critical Care Medicine*. 155, 1356–1361.

Hamada, N., & Abe, N. (2010) Growth Characteristics of Four Fungal Species in Bathrooms. *Biocontrol Science*, *15*(3), 111–115.

Haugland, R., & Vesper, S. (2002) Method of Identifying and Quantifying Specific *Fungi* and *Bacteria.* US Patent 6,387,652.

Henne, K., Kahlisch, L., Brettar, I., & Höfle, M. G. (2012) Analysis of structure and composition of bacterial core communities in mature drinking water biofilms and bulk water of a citywide network in Germany. *Applied and Environmental Microbiology*, *78*(10), 3530–8.

Holinger, E. P., Ross, K. A., Robertson, C. E., Stevens, M. J., Harris, J. K., & Pace, N. R. (2013). Molecular Analysis of Point-of-Use Municipal Drinking Water Microbiology. *Water Research*, *49*, 225–235.

Hwang, C., Ling, F., Andersen, G. L., LeChevallier, M. W., & Liu, W. T. (2012) Microbial community dynamics of an urban drinking water distribution system subjected to

phases of chloramination and chlorination treatments. *Applied and Environmental Microbiology*, *78*(22), 7856–7865.

Ingerson-Mahar, M., & Reid, A. (2012) Microbes in Pipes: The Microbiology of the Water Distribution Systems. *The American Academy of Microbiology* (pp. 1–28). Boulder, Colorado.

Kahana, L. M., Kay, J. M., Yakrus, M., & Waserman, S. (1997) *Mycobacterium avium* complex infection in an immunocompetent young adult related to hot tub exposure. *Chest*, 111, 242–5.

Kanzler, D., Buzina, W., Paulitsch, a, Haas, D., Platzer, S., Marth, E., & Mascher, F. (2008) Occurrence and hygienic relevance of fungi in drinking water. *Mycoses*, *51*(2), 165–169.

Keating, G. A., Mckone, T. E., & Gillett, J. W. (1997) Measured and estimated air concentrations of chloroform in showers: Effects of water temperatures and aerosols. *Atmospheric Environment*, 31(2), 123–130.

Kelley, S. T., Theisen, U., Angenent, L. T., Amand, A. S., & Pace, N. R. (2004) Molecular Analysis of Shower Curtain Biofilm Microbes. *Applied and Environmental Microbiology*, *70*(7), 4187–4192.

Kinney, K.A., Hoisington, A., Maestre, J.P. and King, M.D. (2012) "Characterization of the Fungal Microbiome Aerosolized in a Residential Shower Unit," Presented at Healthy Buildings, Brisbane, AU, July 8-12, 2012.

Kinsey, G. C., Paterson, R. R., & Kelley, J. (1999) Methods for the determination of filamentous fungi in treated and untreated waters. *Journal of Applied Microbiology Symposium*, Supplement 85, 214S-224S.

Krsek, M., & Wellington, E. M. (1999) Comparison of different methods for the isolation and purification of total community DNA from soil. *Journal of Microbiological Methods*, 39(1), 1–16.

Lazarecie, C., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Østerås, M., Schrenzel, J., & François, P. (2009) Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *Journal of Microbiological Methods*, 79, 266-271.

Lechevallier, M. W., Lowry, C. D., Lee, R. G., & Gibbon, D. L. (1993) Examining the relationship between iron corrosion and the disinfection of biofilm bacteria. *Journal of the American Water Works Association*, 85 (7), 111−123.

Lu, P., Chen, C., Wang, Q., Wang, Z., Zhang, X., & Xie, S. (2013) Phylogenetic diversity of microbial communities in real drinking water distribution systems. *Biotechnology and Bioprocess Engineering*, 18(1), 119–124.

Makin, T., & Hart, C. A. (1990) The efficacy of control measures for eradicating legionellae in showers. *Journal of Hospital Infection*, 16, 1–7.

Marras, T. K., Wallace, R. J., Koth, L. L., Stulbarg, M. S., Cowl, C. T., & Daley, C. L. (2005) Hypersensitivity pneumonitis reaction to *Mycobacterium avium* in household water. *Chest*, 127, 664–671.

Marthi, B., Fieland, V. P., Walter, M., & Seidler, R. J. (1990) Survival of bacteria during aerosolization. *Applied and Environmental Microbiology*, *56*(11), 3463-3467.

McFarland, A. R., Haglund, J. S., King, M. D., Hu, S., Phull, M. S., Moncla, B. W., & Seo, Y. (2010) Wetted Wall Cyclones for Bioaerosol Sampling. *Aerosol Science and Technology*, 44(4), 241–252.

Metzger, W. J., Patterson, R., Fink, J., Semerdjian, R., & Roberts, M. (1976) Sauna-takers disease. Hypersensitivity due to contaminated water in a home sauna. *Journal of the American Medical Association*, 236, 2209–2211.

Morton, S. C., Zhang, Y., & Edwards, M. A. (2005) Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. *Water Research*, 39 (13), 2883−2892.

Muittari, A., Kuusisto, P., Virtanen, P., Sovijärvi, A., Grönroos, P., Harmoinen, A., Antila, P., & Kellomäki, L. (1980) An epidemic of extrinsic allergic alvaeolitis caused by tap water. *Clinical Allergy*, 10, 77-90.

Nightingale, S. D., Byrd, L. T., Southern, P. M., Jockusch, J. D., Cal, S. X., & Wynne, B. A. (1992) Incidence of *Mycobacterium avium*-intracellulare complex bacteremia in human immunodeficiency virus-positive patients. *Journal of Infectious Disease,* 165, 1082–1085.

Oliveira, B. R., Barreto Crespo, M. T., San Romão, M. V., Benoliel, M. J., Samson, R. A., & Pereira, V. J. (2013) New insights concerning the occurrence of fungi in water sources and their potential pathogenicity. *Water Research*, 47, 6338-6347.

Owen, M., Ensor, D., & Sparks, L. (1992) Airborne particle sizes and sources found in indoor air. *Atmospheric Environment*, 26(12), 2149–2162.

Panwalker, A. P., & E. Fuhse. 1986. Nosocomial *Mycobacterium gordonae* pseudo infection from contaminated ice machines. *American Journal of Infection Control*, 7, 67–70.

Perkins, S. D., Mayfield, J., Fraser, V., & Angenent, L. T. (2009) Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. *Applied and Environmental Microbiology*, *75*(16), 5363–5372.

Pinto, A. J., Xi, C., & Raskin, L. (2012) Bacterial community structure in the drinking water microbiome is governed by filtration processes. *Environmental Science & Technology*, *46*(16), 8851–9.

Portaels, F. (1995) Epidemiology of mycobacterial diseases. *Clinics in Dermatology,* 13, 207-222.

Porter, T. M. & Golding, G. B. (2011) Are similarity- or phylogeny-based methods more appropriate for classifying internal transcribed spacer (ITS) metagenomic amplicons? New Phytologist, 192, 775-782.

Prince, D. S., Peterson, D. D., Steiner, R. M., Gottlieb, J. E., Scott, R., Israel, H. L., Figueroa, W. G., & Fish, J. E. (1989) Infection with *Mycobacterium avium* complex in patients without predisposing conditions. *The New England Journal of Medicine*, 321, 863–868.

Reponen, T., Willeke, K., Uleviciusj, V., Reponen, A., & Grinshpun, S. A. (1996) Effect of relative humidity on the aerodymanic diameter and respiratory deposition of fungal spores. *Atmospheric Environment*, *30*(23), 3967–3974.

Rose C. S., Martyny, J. W , Newman, L. S., Milton, D. K., King, T.E., Beebe, J. L., et al. (1998) "Lifeguard lung": endemic granulomatous pneumonitis in an indoor swimming pool. *American Journal of Public Health*, 88, 1795–800.

Rutala, W. A. & Weber, D. J. (2008) Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008. US Department of Health & Human Services Center for Disease Control. 1–158.

Salah, I. B. & Drancourt, M. (2010) Surviving within the amoebal exocyst: the *Mycobacterium* avium complex paradigm. *BMC Microbiology*, 10, 1471–1780.

Salo, P. M., Arbes, S. J., Sever, M., Jaramillo, R., Cohn, R. D., London, S. J., & Zeldin, D. C. (2006) Exposure to *Alternaria alternata* in US homes is associated with asthma symptoms. *The Journal of Allergy and Clinical Immunology*, *118*(4), 892–8.

Scanlan, D. J., & West, N. J. (2002) Molecular ecology of the marine cyanobacterial genera *Prochlorococcus* and *Synechococcus*, *FEMS Microbiology Ecology*, 40, 1-12.

Schoen, M. E., & Ashbolt, N. J. (2011) An in-premise model for *Legionella* exposure during showering events. *Water Research*, *45*(18), 5826–36.

Su, H., van Bruggen, A. H. C., Subbarao, K. V., Scherm. (2003) Sporulation of *Bremia lactucae* affected by temperature, relative humidity, and wind in controlled conditions. *Phytopathology*, 94(4), 396-401.

Taylor, R. H., Falkinham III, J. O., Norton, C. D., & LeChevallier, M. W. (2000) Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Applied and Environmental Microbiology*, 1702–1705.

Thomas, R. J., Webber, D., Sellors, W., Collinge, A., Frost, A., Stagg, A. J., Bailey, S. C., Jayasekera, P. N., Taylor, R. R., Eley, S., & Titball, R. W. (2008) Characterization and deposition of respirable large- and small-particle bioaerosols. *Applied and Environmental Microbiology.* 74(20), 6437–6443.

US EPA. (1989) Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and E. Coli) Final Rule. Federal Register, 54(124), 27544-27568.

US EPA. (2013). National Primary Drinking Water Regulations: Revisions to the Total Coliform Rule; Final Rule. Federal Register, 78(30), 10270-10365.

Vaerewijck, M. J. M., Huys, G., Palomino, J. C., Swings, J., & Portaels, F. (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiology Reviews*, 29(5), 911-934.

Van der Wielen, P. W. J. J., & van der Kooij, D. (2013) Nontuberculous mycobacteria, fungi, and opportunistic pathogens in unchlorinated drinking water in The Netherlands. *Applied and Environmental Microbiology*, 79(3), 825–34.

Vesper, S. J., Haugland, R. A., Rogers, M. E., & Neely, A. N. (2007) Opportunistic Aspergillus pathogens measured in home and hospital tap water by quantitative PCR (QPCR). *Journal of Water and Health*, 5(3), 427–431.

von Reyn, C. F., Maslow, J. N., Barber, T. W., Falkinham III, J. O., & Arbeit, R. D. (1994) Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet*, 343, 1137–1141.

Wang, H., Masters, S., Hong, Y., Stallings, J., Falkinham, J. O., Edwards, M. a, & Pruden, A. (2012) Effect of disinfectant, water age, and pipe material on occurrence and persistence of *Legionella*, mycobacteria, *Pseudomonas aeruginosa*, and two amoebas. *Environmental Science & Technology*, *46*(21), 11566–74.

Wang, Y. & Qian, P. Y. (2009) Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One*, 4(10), e7401.

Whiley, H., Keegan, a, Giglio, S., & Bentham, R. (2012). *Mycobacterium avium* complex--the role of potable water in disease transmission. *Journal of Applied Microbiology*, *113*(2), 223–32.

White, T., Burns, T., Lee, S., & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, 315–322. *In:* Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T.J. (ed), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

White, C., Tancos, M., & Lytle, D. A. (2011) Microbial community profile of a lead service line removed from a drinking water distribution system. *Applied and Environmental Microbiology*, 77(15), 5557–5561.

Wilkes, C. R., Mason, A. D., & Hern, S. C. (2005) Probability distributions for showering and bathing water-use behavior for various U.S. subpopulations. *Risk Analysis*, 25(2), 317-337.

Wolinsky, E. (1979) Nontuberculous mycobacteria and associated diseases. *American Review of Respiratory Disease.* 119, 107–159.

Xu, X., & Weisel, C. P. (2003) Inhalation exposure to haloacetic acids and haloketones during showering. *Environmental Science & Technology*, 37(3), 569–576.

Yokota, A., Fujii, T., & Goto, K. (2007) *Alicyclobacillus*: thermophilic acidophilic bacilli. Tokyo: Springer. ISBN 978-4-431-69849-4.

Zhou, Y., Benson, J. M., Irvin, C., Irshad, H., & Cheng, Y.-S. (2007) Particle size distribution and inhalation dose of shower water under selected operating conditions. *Inhalation Toxicology*, *19*(4), 333–42.